INTERSPECIFIC HYBRIDIZATION AMONG FOUR SPECIES OF THE GENUS VIGNA SAVI

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ABSTRACT

Crosses with three lines of mung bean (V. radiata) and two lines each of adzuki bean (V. angularis), black gram (V. mungo) and rice bean (V. umbellata) were made in order to determine the barriers to hybridization between species, and to assess the possibility of overcoming these barriers. This would provide useful information for exploiting wide germplasm resources in breeding these valuable pulse crops.

Reciprocal differences were common in all the species combinations. These differences were observed in pod set, embryo abortion, embryo culture, and seedling lethality. No crosses were successful in both directions.

The results of six interspecific combinations are summarized as follows:

(1) Adzuki bean and black gram was the least successful combination. Although pod set was normal when adzuki bean was used as the female, embryo abortion was complete and no seedlings obtained from embryo culture survived beyond the seedling stage.

(2) Black gram and rice bean was also a very difficult combination to produce. Some seedlings obtained by embryo culture from crosses using black gram as the pistillate parent reached adult stage, but died before flowering.

(3) Adzuki bean and mung bean was a somewhat more successful combination. Hybrids from mung bean produced by embryo culture flowered profusely but were completely sterile. Lack of chromosome pairing, shown by an average of $2.39_{II} + 17.22_{I}$ seems likely to be a principal cause of sterility.

(4) The mung bean and rice bean combination was somewhat easier to make than the last. Embryo abortion is not complete so that some crinckled but viable seeds are produced on mung bean used as the pistillate parent. However, the hybrids, with average chromosome pairing of $0.09_{IV} + 4.69_{II} + 12.26_{I}$, are still completely sterile. Colchicine-induced amphidiploids showed regular meiosis with 22 bivalents and greatly improved fertility, indicating that at least in this combination much of the sterility of the diploid hybrid is caused by chromosomal factors. However, low pod set and defective seeds in the amphidiploid indicate that some adverse genic interactions are still found.

(5) The mung bean and black gram combination (the former used as the pistillate parent) produced hybrid seeds with good germination. However, the F_1 hybrids were only partially fertile. One quadrivalent in 37% of the cells examined indicates that one reciprocal translocation has occurred during chromosome differentiation of these two species. Segregation for morphological characters and the appearance of highly fertile individuals in the F_2 and BC_1 demonstrate the possibility of gene exchange between these two species.

(6) The adzuki bean and rice bean combination obtained by culture of embryos from rice bean used as the pistillate parent was highly fertile with completely normal bivalent formation. Segregation for morphological characters and high fertility in the F_2 and BC_1 indicate the possibility of gene exchange between these species also.

On the basis of crossability relationships, and on the assumption that cytoplasm which prevents the development of a hybrid embryo is

the evolved cytoplasm, while cytoplasm which permits development of a hybrid embryo is the original type, evolutionary relationships of these four species are proposed. Mung bean and rice bean are assumed to have been derived independently from a common ancestor, possibly V. radiata var. soblobata which is a wild species. Black gram and adzuki bean are considered to be later derivatives from mung bean and rice bean, respectively. Thus, these four species can be divided into two subgroups, mung bean with black gram and rice bean with adzuki bean, in which the two species of each group retain enough chromosomal homology to allow germplasm exchange within the group.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

LIST OF TABLES

LIST OF TABLES (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

INTRODUCTION

Interspecific hybridization, followed by natural polyploidy, has played an important role in the evolution of flowering plants in nature (Stebbins, 1950, 1971; Barber, 1970; Knoblock, 1972).

Experimental interspecific hybridization is used to study the nature of isolating mechanisms operating between species and to study phylogenetic relationships.

In plant breeding, interspecific hybridization has been used when desired characters are not found in the range of variation already present in the species. Other advantages of interspecific hybridization could be the possibility of producing hybrids with higher mutation rates (Stebbins, 1969), increased environmental adaptiveness (Stebbins, 1969) or combinations of new characters not present in either parental species (Rick, 1967; Knoblock, 1972; Harlan, 1976). Therefore, interspecific hybridization is a promising tool by which a plant breeder can create broader genetic diversity.

The oriental species of beans, Vigna radiata (L.) Wilczek (mung bean), V. mungo (L.) Hepper (urd or black gram), V. angularis (Willd.) Ohwi and Ohashi (adzuki bean), and V. umbellata (Thunb.) Ohwi and Ohashi (rice bean), are major pulse crops in a large part of Asia, the eastern part of Africa, and some other parts of the world. Although the taxonomy of this group has undergone many changes, they have always been treated as a related group and referred to as the 'mung' group or oriental beans. Characteristics which distinguish them from other beans are their Asian origin and their yellow flowers.

The purpose of this study is to determine the nature of reproductive barriers existing between these four species, and to assess the possibility of overcoming the barriers, which would allow the exploitation of germplasm resources beyond individual species boundaries in breeding this group of valuable beans.

LITERATURE REVIEW

Taxonomy

The Leguminosae is economically one of the most important groups of flowering plants, providing a variety of food sources, animal feeds and raw materials. Some members are grown as soil building crops or as ornamentals (Leppick, 1966; Purseglove, 1968; Hutchinson, 1969; Heywood, 1971). It is a natural group, easily defined taxonomically by a single character, a special form of fruiting capsule called a 'legume' (Maekawa, 1955; Tuton, 1958).

There is still no general agreement on the treatment of major taxa. Some treatments divided the family Leguminosae into three subfamilies; Caesalpinoideae, Mimosoideae and Lotoideae (syn. Papilionoideae, Faboideae, Papilionatae) (Laurence, 1951). The Swartzioideae is sometimes separated from the Caesalpinoideae and appears as a fourth subfamily (Heywood, 1971). Hutchinson (1973), however, elevates the Leguminosae to an order, Leguminales, comprising three separate families; Caesalpinaceae, Mimosaceae and Fabaceae (syn. Papilionaceae).

The Fabaceae, due to the distinctive floral structure of a usually gamosepalous calyx and papilionate corolla, is easily distinguished from the members of other subgroups. The Phaseolineae, a tribe of Fabaceae, includes all the cultivated beans. Members belonging to this tribe have bearded styles and beaked or spirally twisted keels, and usually have stipels, inflorescences with swollen nodes and uniform anthers.

There has also been a great deal of confusion in the minor taxa of oriental beans, chiefly because of the shape of their keels. The keel of these beans is curved like that of some members of the genus Vigna and slightly twisted spirally at the same time. The spirally coiled keel is a distinctive characteristic of the genus Phaseolus.

Oriental beans had been generally included in Phaseolus L. until Verdicourt (1970) revised the taxonomy of this group of beans to put them into the subgenus Ceratotropis (Piper) Verdc. of the genus Vigna Savi. The Agricultural Research Service of U.S.D.A. recognized this revision (Gunn, 1973). The basis of Verdicourt's revision is that these beans have a medifixed stipule, septate pods, and markedly reticulate pollen compared to the basifixed stipules, non-septate pods, and pollen grains with either no obvious sculpture or only a very fine reticulation in Phaseolus beans. In addition to morphological characters, Verdicourt also relied on free amino acid patterns and serological reactions which confirm that this group is related more closely to the other members of Vigna than to Phaseolus.

This group of beans differs from other members of Vigna by having the stipule distinctly peltate and the style beaked beyond the stigma with the thickened part strongly curved.

The botanical names revised by Verdicourt are Vigna angularis (Willd.) Ohwi and Ohashi for adzuki bean, V. mungo (L.) Hepper for black gram, V. radiata (L.) Wilczek for mung bean, and V. umbellata (Thunb.) Ohwi and Ohashi for rice bean. Hereafter common names will be used to alleviate confusion.

The synonyms frequently appearing in the literature are; Phaseolus angularis, Dolichos angularis and Azukia angularis for adzuki bean; P. mungo, A. mungo and Rudua mungo for black gram; P. aureus, P. radiatus, A. radiata and R. aurea for mung bean; and P. calcaratus, P. pubescens, P. ricciardinus, D. umbellata, V. calcarata, and A. umbellata for rice bean.

Cytology

According to Darlington and Janaki-Ammal (1945), chromosome numbers for these four species were first reported as 2n = 22 by Karpechenko in 1925, but Rau reported 2n = 24 for black gram in 1929. Later Sen and Ghosh (1960), Dana (1966a), De and Krishnan (1966a) and Chaurasia and Sharma (1974) all reported 2n = 22 for black gram. Kaul (1970) occasionally observed precocious separation of one bivalent in meiosis of mung bean that was regular otherwise.

Interspecific hybridizations involving the four Vigna species

The first reported interspecific hybridization among these species was between mung bean and black gram (Sen and Ghosh, 1960). Other studies on crosses between mung bean and black gram were conducted by Boling et al. (1961), Khanna et al. (1962), Dana (1966a), De and Krishnan (1966b), AVRDC (Anonymous, 1974), and Singh and Singh (1975). The objective of making this cross was to combine the agronomic and horticultural qualities and early maturity of mung bean with the high yield potential, high content of sulfur-bearing amino acids, plant vigor, resistance to many kinds of diseases and insects, and tolerance to adverse weather conditions of black gram.

Partially filled viable seeds were produced only when mung bean was used as the pistillate parent. The reciprocal cross failed. Hybrid lethality was observed at all stages of development from germination to flowering. Lethality, weakness and sterility continued to occur in the F_2 generation. The F_2 and backcross to mung bean produced plants with combined characters of both parents. This implies the possibility of germplasm interchange between these two species.

In the F_1 plants the average number of bivalents at metaphase I was 9 to 10. Meiotic irregularities such as univalents, quadrivalents, laggards, improper congregation of the chromosomes on the equatorial plates and precocious movements of chromosomes were often observed. Quadrivalents occurred at the frequency of 0.2 per cell, which suggested that a reciprocal translocation occurred (Dana, 1966a). Based on pachytene analysis De and Krishnan (1966b) found that a terminal deletion and an interstitial duplication were also involved.

Hybrid inviability, weakness, sterility and breakdown are considered to be reproductive barriers between these two species (Dana, 1966a). However, the strength of the isolating mechanisms varies, depending upon the parental genotypes used in producing the hybrids. Therefore, mung bean and black gram are considered to be closely related species which differ chromosomally by one major reciprocal translocation and several minor modifications. Dana (1966a) gave the genomic notation AA for the two species.

Dana (1966b) also reported crossing mung bean and rice bean. The objective of this cross was to transfer the hypogeal germination of rice bean into mung bean. Partially filled viable seeds were

produced only in the cross, mung bean X rice bean. In the reciprocal cross, young pods dropped in the early stages of development. Meiosis of F_1 plants was highly irregular with univalents, bridges, precocious separation of chromosomes, and improper arrangement of chromosomes on equatorial plates. The modal chromosome pairing was $8_{II} + 6_I$. The F_1 had only 1.1% stainable pollen and was completely sterile. A colchicine-induced amphidiploid showed gigas characteristics with much increased stainable pollen (70.7%) and many pods with viable seeds. The isolating barriers existing between these two species were considered by Dana to be hybrid inviability and sterility. The hybrid sterility was interpreted as chromosomal with a possible genic cause involved. The genomic notations AA for mung bean and AlAl for rice bean were proposed.

Hybrids of mung bean X rice bean were also produced by Sawa (1973) from cultured embryos. This cross was made for the purpose of transferring the cyst-nematode resistance of the mung bean into adzuki bean. The bean which he originally considered to be a semi-wild relative of adzuki bean, however, was later identified as a local variety of rice bean (Sawa, 1974). The F_1 was very vigorous but completely seed sterile. Abnormal polyspory in microsporogenesis was considered to be responsible for the hybrid sterility. Stainable pollen in the colchicine-induced amphidiploids was 58 to 88% compared to 2.5% in the F_1. The average seed set was 24.5% and 22 bivalents were counted at metaphase I of the C_2 plants.

In their mung bean breeding program in AVRDC (Anonymous, 1974, 1975), mung bean X rice bean hybrids were produced. The specific

objective is to introduce the resistance to diseases and insects and the high methionine content of the rice bean into mung bean. The F_1 plants were sterile but produced a single pod with four seeds. The F_2 plants were identified as spontaneous amphidiploids (Baker, personal communication). Reciprocal backcrosses to both parental species were tried without success.

Al-Yasiri and Coyne (1966) classified the cross black gram X rice bean as a partially compatible cross in which pods collapse in the early stages of development, and the reciprocal cross as incompatible in which no pods set.

The only success with a cross between thse two species was reported by Biswas and Dana (1975). Hybrids were raised by culturing the embryonic axes of the dried shrivelled seeds produced only in black gram X rice bean. Differential compatibility determined by the female genotypes was noticed. The hybrid plants were weak and stunted in growth during the winter but grew vigorously in summer. In the F_1, stainable pollen was only 0.7% on the average and no pod set was noticed. The number of bivalents in the F_1, some of which were loosely paired, ranged from 3 to 6 in most of the cells observed. Quadrivalents and bridges were occasionally found. Colchicine-induced amphidiploids produced 81% fertile pollen and mature pods with viable seeds. Hybrid inviability due to incompatibility between embryo and endosperm as well as seedling lethality, hybrid weakness, and hybrid sterility were considered important reproductive barriers between these two species by Biswas and Dana (1975).

The adzuki bean X rice bean cross is partially compatible, while rice bean X adzuki bean is incompatible according to the classification of Al-Yasiri and Coyne (1966). No other record has been found of successful crosses between these two species, except a brief comment by Sawa (1973) that fertile hybrids had been obtained by culturing young embryos from the cross, rice bean X adzuki bean.

There is apparently no record of successful crossing of adzuki bean with black gram or mung bean. The cross, mung bean X adzuki bean, was attempted at AVRDC (Baker, personal communication) without success. Crosses between adzuki bean and black gram in both directions are rated as incompatible by Al-Yasiri and Coyne (1966).

An unidentified natural tetraploid species of Vigna, which has been considered to be of amphidiploid origin (Dana, 1964; Krishnan and De, 1970) and which most likely is the same species identified as Vigna radiata (L.) Wilczek var. glabra (Roxb.) Verdc. by a USDA taxonomist (Swindell et al., 1973), has been crossed with mung bean, rice bean and black gram (Dana, 1964, 1965, 1968; Krishnan and De, 1968a, b).

A hybrid from the cross, mung bean X the natural tetraploid species, was obtained by Dana (1965). The hybrid set pods which shed within 2 to 3 days and the F_1 plant died 14 days after flowering began. Apparently well-developed pods harvested from the reciprocal cross contained only shrivelled nonviable seeds.

Krishnan and De (1968a) crossed a colchicine induced autotetraploid mung bean with the natural tetraploid species. Hybrids were obtained only when the autotetraploid mung bean was used as the female.

The hybrids were completely seed sterile. The average chromosome pairing in the F_1 plants was $15_{II} + 14_I$.

Black gram was crossed with the tetraploid species by Dana (1968). Completely sterile hybrids with 0.83% stainable pollen were obtained only when black gram was used as the female parent. Colchicine induced amphidiploids (allohexaploids) had gigas characteristics, were semi-fertile, and produced a few small pods with viable seeds.

Krishnan and De (1968b) obtained completely sterile hybrids when they crossed a colchicine induced autotetraploid black gram with the tetraploid species. The average chromosome pairing in the F_1 was $0.02_{IV} + 0.41_{III} + 7.77_{II} + 16.15_I$.

Dana (1964) obtained hybrids from the cross of the tetraploid species X rice bean by culturing young ovules. Chromosome pairing in the completely sterile hybrids was $11_{II} + 11_I$ in most of the cells examined. Thus, he concluded that the natural tetraploid species is an amphidiploid with one genome homologous with the genome of rice bean.

Extensive studies were conducted on a cross between mung bean and V. trilobata by Dana (1966c,d) and Dana and Das (1974). V. trilobata (syn. Dolchos trilobatus, Phaseolus trilobatus and P. trilobus) is a forage legume. Hybrids were obtained only when mung bean was used as the female. The hybrids were partially fertile with 31% stainable pollen on the average. In the hybrids 11 bivalents were counted in 39.9% of the cells studied. Meiotic irregularities, such as quadrivalents, precocious separation of chromosomes, failure of chromosomes to congregate on the equatorial plate, bridges, and laggards were often noticed.

Highly fertile amphidiploids were induced artificially from this hybrid by colchicine treatment. Natural amphidiploids were also raised from seeds which set on one fertile branch, which probably originated from somatic chromosome doubling in the meristem of a lateral bud. Natural amphidiploids and triploids were also found among the F_2 progeny of diploid hybrids. Restitution during gametogenesis was thought to be responsible for the production of these polyploids from the diploid hybrid. Meiosis in the amphidiploids was fairly regular with 22 bivalents in most of the cells examined.

The same genomic notation of AA was given to both of these species. Hybrid inviability, weakness, and sterility were considered as the main isolating barriers acting between these two species.

Strand (1943) reported that he obtained fertile progenies from the cross Phaseolus vulgaris X black gram. Kloz et al. (1960), however, questioned this result on the basis of their distant relationship in serological reaction. A positive relationship between the degree of similarity in protein characters and crossability, graft affinity, and geographical origins was postulated. According to Yarnell (1965), Strand in 1950 produced a hybrid seedling from the cross, Phaseolus vulgaris X adzuki bean. The seedling was pale green at first and later turned white. Due to the commonly accepted distant relationship between black gram and adzuki bean and P. vulgaris, and the difficulty in crossing even closely related species, these last two reports, especially the former one, are very questionable.

Embryo abortion

Hybrid inviability is a common phenomenon in interspecific crosses. Embryo abortion is a type of inviability which operates in the early developmental stages of zygotes.

Somatoplastic sterility, in which retarded embryo growth, hyperplasia of the nucellus, and failure of normal development of the conducting elements are considered to be responsible for ovule collapse, was postulated as the cause of hybrid seed failure in species crosses in Nicotiana by Cooper and Brink (1940) and Brink and Cooper (1941). Satina et al. (1950) felt that the formation of ovular tumors in hybrid ovules caused embryo abortion in incompatible species crosses of Datura. In an extensive review on plant genetic tumors, Smith (1972) concluded that ovular tumors in interspecific hybrids which are associated with the failure of hybrid embryos are genetical and the result of the interaction between the different parental contributions. The tumor-prone hybrid plants synthesize or accumulate greater than regulatory amounts of growth-promoting substances such as auxins and cytokinins.

Hybrid embryo abortion is known to occur widely in the legumes. Johansen and Smith (1956) reported that the cause of embryo abortion in the cross, Arachis hyposia X A. diogoi, is due to slow embryo growth, malfunctioning of the endosperm, and failure of endosperm differentiation. Griton and Wierzbica (1975) presumed that the failure of endosperm to develop and function normally leads to embryo starvation in the intergeneric cross of Pisum sativum X Vicia faba. Polytene chromosomes in the suspensor of Phaseolus vulgaris were discovered by

Nagl (1969), which indicates that the suspensor is an area of high metabolic activity. Haq et al. (1973) assumed that the low level of activity of the suspensor region, as shown by low staining ability, has some connection with the retarded development of the endosperm and embryo in the cross, Phaseolus coccineus X P. vulgaris. Furuya and Soma (1957) observed that a high concentration of auxins brought about suppression of growth and proliferation of tumorous tissue in the culture of P. vulgaris embryos.

Methods of counteracting hybrid embryo abortion

Skirm (1942) regarded embryo culture as a promising tool in plant breeding, by which interspecific seed sterility expressed by the abortion of partially developed embryos can be overcome. According to Blakeslee (1945), embryos at or later than the torpedo stage can be excised and cultured.

Young embryos from Phaseolus vulgaris X P. acutifolius, which otherwise abort, were successfully cultured in White's medium by Honma (1955). The older and larger embryos grew faster and sturdier. He also found that a high sucrose concentration in the medium favored root growth while a low concentration favored shoot growth. Al-Yasiri and Coyne (1964) reported that the application of naphthalene acetamide alone or together with potassium gibberellate to the stigma just before pollination delayed embryo abortion in the cross, Phaseolus vulgaris X P. acutifolius, so that the resulting embryos were large enough to be cultured in vitro.

Ibrahim and Coyne (1975) reported that application of White's nutrient solution to the stigmatic surface prior to pollination enhanced

pod set, while detachment of pods from plants prevented embryo abortion in the cross Phaseolus coccineus X P. vulgaris. Pods showing signs of embryo abortion were left on the plant but partially detached by breaking the pedicels, or taken off from the plant and kept in an air-tight plastic bag in a growth chamber. Under these treatments, embryos grew into viable seeds. They considered that inhibiting substances produced in P. coccineus leaves and translocated to the pods were the cause of embryo abortion.

Baker et al. (1973) reported that the immunosuppressant, amino caproic acid, promoted pod set and development in reciprocal crosses between mung bean and rice bean.

Embryo culture has also been employed to produce interspecific hybrids between species belonging to the mung group. Dana (1964) produced hybrids of the tetraploid species X rice bean by culturing young embryos. Sawa (1973) followed Honma's procedure to obtain hybrids from crosses of mung bean X rice bean and rice bean X adzuki bean. Biswas and Dana (1975) obtained hybrids from a black gram X rice bean cross only by culturing the poorly developed embryonic axes dissected out of the water-soaked seeds.

MATERIALS AND METHODS

Parental lines

Three lines of mung bean and two lines each of adzuki bean, black gram, and rice bean were used as parents for this study. The origins and some morphological characteristics are shown in Tables 1, 2 and Figures 1-8. Hereafter, the lines will be referred to by the symbols listed in Table 1. All lines were received from the Western Regional Plant Introduction Station at Pullman, Washington and increased.

Characters such as germination habit, petiole length of primary leaves, and hairiness on the pod clearly divide these species into two groups, mung bean and black gram versus adzuki bean and rice bean. Black gram and mung bean have epigeal germination, sessile primary leaves, and hairy pods while adzuki bean and rice bean have hypogeal germination, long-petioled primary leaves, and glabrous pods.

Black gram can be easily distinguished from mung bean because black gram has upright pods at maturity, thick and smooth pods, and a concave hilum cushion in contrast to radiate or drooping pods, slightly constricted pods, and straight hilum in mung bean. Two of the three mung bean lines and both of the black gram lines were slightly delayed in flowering in the summer. Summer flowering was sparse in black gram, but not in mung bean.

Adzuki bean can be distinguished from rice bean by broadly cordate primary leaves, conspicuously constricted pods, and a straight hilum, in contrast to lanceolate primary leaves, smooth pods, and a concave hilum cushion in rice bean. The photoperiodic response seems to be

Table 1. The origins of nine parental lines of four oriental bean species

Common names	P. I. No.	Symbols	Origins
Adzuki bean	175240	A1	India
	237689	A2	Japan
Black gram	174907	B1	India
	208462	B2	Nepal
Mung bean	200840	M1	Burma
	207504	M2	Afghanistan
	377231	M3	India
Rice bean	276369	R1	India
	322571	R2	Brazil

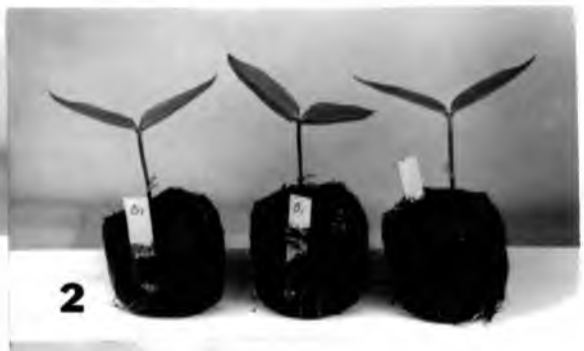
Table 2. Characteristics of lines of oriental bean species used in crosses

Characters	Lines								
	A1	A2	R1	R2	M1	M2	M3	B1	B2
Germination	hypogeal				epigeal				
Primary leaves	long-petiolate				sessile				
Pod	glabrous				hairy				
Shape of primary leaves	broadly cordate		lanceolate						
Pods at maturity	drooping				radiate or drooping			upright	
Constriction of pod	conspicuous		smooth		slight			smooth	
Hilum cushion	not concave		concave		not concave			concave	
Seed color	yellow, purple speckl.	maroon	red	green-yellow	green	grey-green	yellow-green	dark grey and white spotted	
Pod length (cm)	6.1	7.5	8.3	8.4	7.2	6.4	7.5	4.4	4.0
Seed size (mm):									
length	6.2	5.5	7.7	7.1	4.0	3.8	4.3	5.2	4.8
width	5.1	4.2	4.8	4.1	3.4	3.5	3.8	3.9	4.3
Seed weight (mg)	11.4	6.6	11.2	7.5	4.1	4.3	5.0	4.8	5.4
Days to flower:									
winter	32	32	45	41	43	31	30	30	37
summer	30	33	no fl.	45	49	36	32	37	43
Seed number per pod	3.8	5.7	6.4	6.3	11.1	10.2	11.1	4.7	5.2

Figs. 1-4. Seedlings of parental species: 1) adzuki bean (A2); 2) black gram (B1); 3) mung bean (M2); 4) rice bean (R1).

Figs. 5-8. Mature plants of parental species: 5) adzuki bean (A2); 6) black gram (B2); 7) mung bean (M2); 8) rice bean (R2).


1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day-neutral in both adzuki bean lines and in one rice bean line (R2), but the other rice bean line (R1) requires short days to flower.

Plant Inventory number 322571 is listed as adzuki bean. However, the morphological characters (Table 2) as well as crossability with other species (Tables 3, 6, 12 and 22), clearly show that this line is rice bean. This line has lanceolate primary leaves, a smooth pod, and a concave hilum cushion which are specific characteristics of rice bean and used to distinguish rice bean from adzuki bean. Moreover, P.I. 320571 crosses readily with the other line of rice bean, R1 (Table 3), and fails to produce any mature seeds in crosses with the two lines of adzuki bean, A1 and A2 (Table 22). Therefore P.I. 322571 is treated as a line of rice bean, with the symbol R2.

Methods

Cultural conditions: One to three plants were grown in each 20 cm plastic pot in a mixture of 3 parts soil and 1 part vermiculite. Three to 7 grams of Osmocote (14-14-14) was applied three times at 2 week intervals from the time of germination. Plantings of parental lines were made at 2 week intervals in order to have flowers available whenever desired.

Crossing methods: All crosses were made in the greenhouse from 9 to 11 a.m. during the winter. A modification of the mung bean hybridization technique of Boling et al. (1961) was used. Plump buds due to open on the next day were selected as female flowers. These flower buds were opened with sharp-pointed forceps along the suture of the standard, and the standard and wings were pulled down. After that, one-third of the keel was taken away, the pistil and stamens

were exposed, and the ten stamens were removed. The pistil was pollinated immediately by rubbing the stigma with the pollen-laden pistil from an open flower of the desired male parent. After pollination, the wings and standard were replaced in their original position to protect the pollinated pistil from drying out.

The walks and benches of the greenhouse were wet with water before and after the crossing operation in order to keep the humidity high.

Pod setting was recorded 1 week after pollination.

Germination of hybrid seeds: Hybrid seeds were germinated in Jiffy-7 pots, and the seedlings were transplanted in 1 to 2 weeks to plastic pots with the soil mixture.

Embryo culture: Interpsecific hybrid embryos at various developing stages were dissected out and cultured in Vacin and Went medium according to the procedure reported by Sagawa and Valmayor (1966).

Seedlings obtained by embryo culture were transplanted to Jiffy-7 pots from the test tubes when the shoot was at least 2 cm long. After 2 to 5 days under shade, they were moved to the greenhouse and gradually exposed to direct sunlight by decreasing the saran cloth covering over a week-long hardening period. They were then transplanted to plastic pots with the soil mixture.

Pollen fertility: Pollen fertility was estimated from the percentage of pollen grains that were stained uniformly by 0.5% cotton blue in lactophenol. At least 1,500 pollen grains from five flowers were counted for each plant.

Cytology: For cytological observations, flower buds were collected from 5 to 7 p.m. and fixed in a modified Carnoy's fixative of 6 parts

chloroform, 3 parts absolute ethanol and 1 part glacial acetic acid, as used by Carr (1976). Anthers from the buds kept in the fixative for more than 1 week, were squashed in 1% acetocarmine and mounted with Hoyer's mounting medium following the Beeks' Permanent Squash Method (1955).

Colchicine treatment: An attempt to induce amphidiploidy was made by treating the cuttings with colchicine according to Smartt and Haq (1972). Vigorous branches taken from the hybrid plants were treated with colchicine by immersing the cut ends into 0.1% aqueous solution for 6 hours in a shady place. The cuttings were then washed and rooted under a mist propagator for 2 to 3 weeks.

RESULTS AND DISCUSSION

A. Intraspecific Crosses

Crossability

Self and cross pollinations of lines within species were made after emasculation to determine the fertility of female and male gametes and the effectiveness of the crossing technique as well as the seed development following fertilization (Table 3).

There was a highly significant difference in pod set between species, but the differences between female and male parents or between self and cross pollinations within species were not significant except for the two rice bean lines. R2, used as a seed parent, had a higher percentage of pod set (68.8%) than R1 (26.7%) but there was no difference between these two lines as pollen parents.

The difference in pod set between species and between two lines of rice bean is probably due to differences in tolerance to floral injury from emasculation and pollination. The extremely low pod set in adzuki bean is at least partly due to scant pollen production during the winter.

Pods that set also reached maturity, except in A1 X A1, A1 X A2, and R1 X R2 in which one pod in each combination dropped in the early stage of development. Ripe pods harvested from artificial pollinations contained normal seeds, but the seed number per pod was slightly lower than in open-pollinated flowers (cf. Tables 2 and 3).

The results imply that female and male reproductive systems function normally and that there are no post-fertilization obstacles, such

Table 3. Pod and seed set in pollinations within oriental bean species

Parents F	Parents M	No. of flowers pollinated	No. of pods harvested	% pod set	No. of seeds obtained	No. of seeds per pod
A1	A1	23	4	17.4	9	2.3
	A2	23	6	26.1	13	2.2
A2	A1	20	5	25.0	13	2.6
	A2	21	5	23.8	15	3.0
Total		87	20	23.0	50	2.5
B1	B1	22	9	40.9	39	4.3
	B2	26	7	26.9	29	4.1
B2	B1	20	6	30.0	29	4.8
	B2	20	6	30.0	31	5.2
Total		88	28	31.8	128	4.6
M1	M1	19	13	68.4	119	9.2
	M2	20	12	60.0	107	9.2
	M3	21	15	71.4	148	9.9
M2	M1	20	15	75.0	116	7.7
	M2	19	9	47.4	86	9.6
	M3	20	13	65.0	120	9.2
M3	M1	20	7	35.0	71	10.1
	M2	23	16	69.6	156	9.8
	M3	22	13	59.1	131	10.1
Total		184	113	61.4	1,054	9.3
R1	R1	30	11	36.7	52	4.7
	R2	30	5	16.7	33	6.6
R2	R1	32	19	59.4	106	5.6
	R2	22	16	72.7	78	4.9
Total		114	51	44.7	269	5.3

X^2- test for pod set

Comparison	Self vs. cross pollination	Between lines as receptor	Between lines as pollinator
Within species			
Adzuki bean	0.57^{ns}	0.15^{ns}	0.36^{ns}
Black gram	0.87^{ns}	0.18^{ns}	0.87^{ns}
Mung bean	0.17^{ns}	1.07^{ns}	0.34^{ns}
Rice bean	39.70**	15.90**	0.72^{ns}
Between species	20.80**		

**significant at 1% level; ns = not significant.

as embryo abortion, to normal seed development in self-pollinations or interline crosses within the species.

Ten flowers of each of the nine parental lines were left emasculated without pollination to study the possibility of parthenocarpic fruit development. Apparently, parthenogenesis does not occur in these species since fruit develoment was not observed from flowers left emasculated.

Meiosis

All of the parental species and interline hybrids within species regularly formed 11 bivalents at first meiotic metaphase followed by normal microsporogenesis. A lack of synchronization in anaphasic chromosome disjunction, with precocious separation of one to two pairs of chromosomes was a common phenomenon in these species (Fig. 9).

B. Interspecific Crosses

1. Crosses between adzuki bean and black gram

Crossability

In crosses of adzuki bean X black gram, there was a singificant difference in pod set between the two lines of adzuki bean. On A1 9 pods out of 70 (12.9%) set, while on A2 only 2 out of 76 (2.7%) set (Table 4). The difference between the two black gram lines used as pollen parents was not significant. All the pods shed within 12 days after pollination. Pod growth was much slower than in open-pollinated ones and barely reached 2.5 cm in length before shedding. Loss of turgidity and yellowing indicated the beginning of pod abortion. The embryos at the time of pod abortion were large enough to recognize and remove.

Fig. 9. Chromosome pairing at metaphase I of a parental species (R2), showing 11 bivalents with precocious separation of one bivalent X1,200.

Fig. 10. The hybrid of black gram (B2) X rice bean (R1) obtained by embryo culture.

Figs. 11-12. F_1 hybrids of mung bean (M3) X adzuki bean (A1) obtained by embryo culture: 11) seedlings; 12) mature plant.

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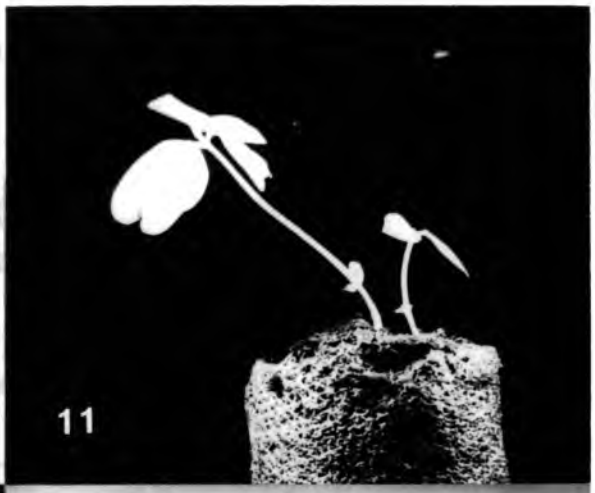

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Table 4. Crossability between adzuki bean and black gram

Parents		No. of flowers crossed	No. of pods set	% pod set
Female	Male			
A1	B1	33	5	15.2
	B2	37	4	10.8
Total		70	9	12.9
A2	B1	39	0	0.0
	B2	37	2	5.4
Total		76	2	2.6
B1	A1	44	5	11.4
	A2	35	9	25.7
Total		79	14	17.7
B2	A1	28	5	17.9
	A2	30	6	20.0
Total		58	11	19.0

X^2- test for pod set

Comparison	X^2 value
Between A X B and B X A	4.46*
Within A X B	16.53**
between A	6.76**
between B	0.09^{ns}
Within B X A	5.63^{ns}
between B	0.04^{ns}
between A	2.28^{ns}

X^2 value significant at 5% (*) or 1% (**); ns = not significant.

The crosses of black gram X adzuki bean showed no differences in pod set between the two lines of black gram or between the two adzuki bean lines used as pollen parents. Pod growth was slightly retarded and abortion usually occurred between 2 and 4 weeks. Embryos at the time of pod abortion were fairly large but slightly deformed.

A difference in crossability according to the direction of hybridization is clearly shown in crosses between these two species. A higher per cent pod set (18.3%) was found when black gram was used as the female than when adzuki bean was (7.5%). Pod set on adzuki bean was markedly lower than in intraspecific pollinations. Black gram also set fewer pods. However, the scant pollen of adzuki bean might be responsible for this low set. Pod abortion was delayed when black gram was used as the female. Pods sometimes lasted almost to the mature stage, while pods aborted within 2 weeks when adzuki bean was used as the female.

Embryo culture

From three embryos from a 9-day old green pod of an A1 X B1 cross, two etiolated seedlings developed which then died. The other embryo formed only roots and callus. Three embryos from a 10-day old dropped pod of the cross A2 X B2 failed to show any sign of growth (Table 5).

Out of 16 embryos from the cross B1 X A2 cultured at various ages between 13 to 26 days, seven grew into normal-appearing seedlings, two showed poor embryonic growth with callus, two formed black callus, and five did not grow at all (Table 5). The green, normal-appearing seedlings started to show signs of wilting 1 to 3 weeks after they were

Table 5. Results of culturing hybrid embryos from crosses between adzuki bean and black gram

Parents		Age of embryo (days)	State of pod	No. of embryos	Results
Female	Male				
A1	B1	9	green	3	2 etiolated seedlings 1 only roots and callus
A2	B2	10	dropped	3	no growth
B1	A1	16	green	1	no growth
B1	A2	13	green	6	2 black callus 4 no growth
		14	green	1	seedling died
		15	green	3	3 seedlings died
		16	green	3	2 poor embryonic growth with callus 1 no growth
		26	dried	3	1 3 plantlets attached, died 2 seedlings died
B2	A2	10	green	1	no growth
		14	green	1	no growth

transplanted to soil pots and eventually died. The hybridity of the seedlings was evident from the short petioled ovate primary leaves. The root systems were found to be poorly developed. In the cross B2 X A2, two embryos were cultured, one 10 days old and the other 14 days old; neither showed any sign of growth.

Poor development of the root systems was suspected to be a primary cause of the seedling mortality, since the seedlings from the cross A1 X B2 showed no other symptoms beside poor root development.

McLean (1946) successfully secured hybrid seedlings containing shoots but no roots from Datura species crosses by grafting them on young vigorous plants of a pure species. Honma (1955) reported that a high sucrose concentration in the embryo culture medium favored root growth while a low concentration favored top growth in the culture of hybrid embryos from the species cross Phaseolus vulgaris X P. acutifolius. Survival of these hybrids could possibly be attained by modifying the culture media so as to encourage better root growth or by grafting them on seedlings of either parental species.

Because of the failure of hybrids between adzuki bean and black gram to grow, these two species are assumed to be the most distantly related of the four species considered herein.

2. Crosses between black gram and rice bean

Crossability

When black gram was used as the female, 69 pods (43.1%) set from 160 flowers pollinated (Table 6). The hybrid pods developed normally and reached maturity. The ripe pods contained two kinds of seeds;

Table 6. Crossability between black gram and rice bean

Parents		No. of flowers crossed	Pod set		No. of pods harvested	No. of seeds obtained	
Female	Male		No.	%		Empty	Filled
B1	R1	44	22	50.0	14	0	43
	R2	39	12	30.8	12	8	32
Total		83	34	41.0	26	8	75
B2	R1	40	25	62.5	16	5	48
	R2	37	10	27.0	8	9	17
Total		77	35	45.5	24	14	65
R1	B1	45	2	4.4	All of the pods shed within 3 weeks.		
	B2	45	2	4.4			
Total		90	4	4.4			
R2	B1	40	0				
	B2	45	0				
Total		85	0				

X^2- test for pod set

Comparison	X^2 value
B X R vs. R X B	36.73**
Within B X R	19.57**
between B	0.23ns
between R	8.59**
Within R X B	8.88*
between R	4.44*
between B	0.004ns

X^2 value significant at 5% (*) or 1% (**); ns = not significant.

small, undeveloped ones and full-size ones which were slightly crinkled. When the slightly crinkled seeds were soaked in water, they appeared normal but the cotyledons were highly distorted and the embryonic axes were poorly developed. Neither kind of seed germinated.

When rice bean was used as female, only 4 pods out of 175 (2.3%) set. Pods grew very slowly and wilted 3 weeks after pollination.

In addition to the reciprocal difference in crossability between these two species, a difference between rice bean lines on pod set was clearly shown. Pod set on black gram was normal when pollinated by R2 but significantly decreased when pollinated by R1. R1 also failed to set any pods when pollinated by black gram pollen while R2 set a few pods.

Embryo culture

From the crosses using black gram as female, 34 hybrid embryos were cultured. Fourteen embryos were taken from 10- to 14-day old pods and 20 from mature pods. Nine apparently good seedlings were obtained; 3 from young pods and 6 from mature pods in the cross B2 X R1. The embryo culture results are listed in Table 7. Of the nine apparently good seedlings, seven died during early seedling stages and only two very weak dwarf plants survived with arrested growth for more than 3 months, and then died before flowering (Fig. 10).

From crosses using rice bean as female, one embryo from an 8-day old green pod and five embryos from a 16-day old wilting pod were cultured. Of these, three embryos formed callus without embryonic development and the other three embryos did not grow at all.

Table 7. Results of culturing hybrid embryos from crosses between black gram and rice bean

Parents		Age of embryo (days)	State of pod	No. of embryos	Results
Female	Male				
B1	R1	10	green	3	1 deformed leaves and callus 2 callus
		22	ripening	2	1 seedling with shoot tip highly coiled 1 seedling died
		28	ripe	3	1 primary leaves only 2 no growth
B2	R1	15	green	6	1 died after 3 months 2 seedlings died 3 no growth
		18	green	5	3 only shoots with callus 2 no growth
		19	ripening	1	no growth
		20	ripening	3	1 seedling died 2 callus
		21	ripening	4	1 etiolated shoot, poor roots and callus 1 seedling died 1 only hypocotyl elongated 1 no growth
		24	ripe	2	no growth
		26	ripe	2	no growth
		27	ripe	3	2 seedlings died 1 died after 3 months
R1	B2	8	green	1	no growth
		16	wilting	5	3 callus 2 no growth

Therefore, embryo culture might be a tool for circumventing the barriers between these two species of embryo abortion and germination failure only when black gram is used as the female parent. Hybrid weakness and inviability are also strong barriers. The extremely weak plants with stunted growth never became vigorous. It is possible that the death of the hybrid plants from this particular cross could be prevented by growing them under a more favorable cultural environment.

Biswas and Dana (1975) reported that they produced hybrids by culturing embryonic axes of mature seeds from a certain black gram X rice bean cross. The hybrid plants, which were weak and stunted in growth during the winter became vigorous in the summer. Amphidiploids induced by chromosome doubling of the completely sterile hybrids produced a few pods with mature seeds. Therefore, growing the hybrid plants under long day conditions to encourage vegetative growth, and trying other parental lines in hybridization might be effective methods to secure hybrid plants that reach the flowering stage.

The reciprocal cross, rice bean X black gram, is not likely to be successful because of a strong barrier to fertilization as well as complete embryo abortion.

Morphology of the hybrid

The hybrid origin of the seedling obtained by embryo culture was confirmed by their germination habit and the petiole length of the primary leaves (Table 8). Germination is epigeal in black gram and hypogeal in rice bean (See Figs. 2 and 3, and Table 2). The cotyledons of the F_1 seedlings remained at or a little above the soil surface without further elongation of the hypocotyl, intermediate between the

Table 8. Morphological comparison of black gram (B2), rice bean (R1), and the hybrid between them

Characters	Black gram (female)	Hybrid	Rice bean (male)
Germination habit	epigeal	intermediate	hypogeal
Petiole length of primary leaves	sessile	short	long
Petiole color	purple	light purple	green
Margin of leaflets	entire	entire	often lobed

parents. The primary leaves are sessile in black gram and long-petiolate in rice bean. The primary leaves of the F_1 seedlings had short petioles, again intermediate between the parents. The petiole color in B2 is purple and green in R1, while that of the F_1 hybrids was intermediate or light purple. The margin of the trifoliate leaflets was entire in the F_1 hybrids, similar to black gram, not lobed as in rice bean.

3. Crosses between adzuki bean and mung bean

Crossability

When adzuki bean was used as the female parent, there were significant differences in pod set between adzuki bean lines (Table 9). A1 set 33 pods out of 105 (31.4%) and A2 set only 7 pods out of 105 (6.7%). Differences between the mung bean pollen parents were not significant. Pod growth ceased in approximately 10 days when pod length reached 1.5 - 2.0 cm. Pods remained green for as much as 3 weeks longer without further development. Embryos barely reached the heart shape stage before they started to degenerate.

When mung bean was used as the pistillate parent, pod set was significantly lower on one line of mung bean. Pod set was only 11.3% on M1 as compared to approximately 30% on M2 and M3. Differences between the adzuki bean pollen parents were not significant. Pods grew slowly and usually shed in 1 to 3 weeks after pollination. Embryos older than 1 week were fairly large and appeared normal.

No reciprocal difference in pod set was found in these crosses. However, pod set on mung bean was less than the pod set from

Table 9. Crossability between adzuki bean and mung bean

Parents		No. of flowers crossed	No. of pods set	% pod set
Female	Male			
A1	M1	36	11	30.6
	M2	34	12	35.3
	M3	35	10	28.6
Total		105	33	31.4
A2	M1	43	2	4.7
	M2	31	1	3.2
	M3	31	4	12.9
Total		105	7	6.7
M1	A1	30	4	13.3
	A2	50	5	10.0
Total		80	9	11.3
M2	A1	34	8	23.5
	A2	34	13	38.2
Total		68	21	30.9
M3	A1	33	8	24.2
	A2	30	11	36.7
Total		63	19	30.2

X^2- test for pod set

Comparison	X^2 value
A X M vs. M X A	0.41^{ns}
Within A X M	51.16**
between A	16.09**
between M	0.64^{ns}
Within M X A	27.65**
between M	10.28**
between A	0.50*

X^2 value significant at 5% (*) or 1% (**); ns = not significant.

intraspecific pollinations while pod set on adzuki bean was similar in both kinds of pollinations. Clear reciprocal differences were found in pod growth, embryo development, and the time of embryo abortion on the two parental species.

Embryo culture

From adzuki bean pods 11 embryos or whole ovules 7 to 21 days old were cultured (Table 10). Of these, only one embryo developed into an etiolated seedling which soon died and another embryo formed only callus while nine embryos showed no growth at all. Embryos or ovules older than 10 days did not show any sign of growth. Therefore, embryo abortion must precede the suspension of pod growth which usually occurs around 10 days after pollination.

From mung bean pods, 26 embryos 9 to 19 days old were cultured and 12 seedlings were obtained, of which 2 were etiolated and soon died and the rest were apparently normal. During the early stages eight seedlings with poor roots died, but two hybrid plants reached flowering stage.

These two hybrid plants were cultured from 19-day old embryos taken from a wilting pod of the cross M3 X A1. Thus, embryo abortion in the mung bean X adzuki bean cross is apparently delayed until the pod collapses, and hybrids can be secured by culturing the young embryos from some parental line combinations.

Morphology of the hybrid

The hybrids showed an intermediate germination habit, the epicotyl color of the male parent, and intermediate petiole length, and

Table 10. Results of culturing hybrid embryos from crosses between adzuki bean and mung bean

Parents		Age of embryo (days)	State of pod	No. of embryos	Results
Female	Male				
A1	M1	7	green	1	etiolated seedling
		10	green	1	no growth
		11	dropped	2	no growth
		16	green	1	no growth
A1	M2	8	green	2	1 callus 1 no growth
		11	green	1	no growth
		13	green	2	no growth
A1	M3	21	wilting	1	no growth
M1	A1	11	dropped	8	no growth
M1	A2	12	green	6	6 seedlings died
		17	wilting	4	2 etiolated seedlings 2 no growth
M2	A2	12	dropped	1	no growth
M3	A1	19	wilting	2	2 mature plants
M3	A2	9	green	5	2 seedlings died 3 no growth

shape of the primary leaves (Fig. 11). Germination is epigeal in mung bean and hypogeal in adzuki bean while the cotyledons of the hybrids rise slightly above the soil surface without further elongation of the hypocotyl. The epicotyl color was purple in M3 but light green in the F_1, resembling the paternal parent, adzuki bean. The primary leaves are sessile and lanceolate in mung bean, but long petiolate and broadly cordate in adzuki bean. The hybrids had ovate and short petioled primary leaves.

The leaf color of the hybrid was dark green and the young pods were hairy, both similar to mung bean, while the trifoliate leaflet margins were lobed as in adzuki bean (Table 11 and Fig. 12).

Fertility of the hybrid

In the F_1 hybrids all pods set from open-pollinated flowers and from controlled selfings were shed in 10 days. Backcrosses of 20 flowers each of all four possible combinations were made. Backcrosses where parental species were used as the females failed to set any pods. However, in the backcrosses with the F_1 as the female parent a few pods set, which dropped within 2 weeks after pollination. Hybrid sterility, which is complete, is considered to be an absolute barrier to hybridization between these two species, at least at the diploid level.

Induction of juvenile shoots, which could be used for vegetative propagation or colchicine treatment, was attempted by applying fertilizer, long-day treatment and cutting back of the main stems. None of the treatments were effective and the plants died.

Table 11. Morphological comparison of mung bean (M3), adzuki bean (A1), and the hybrid between them

Characters	Mung bean (female)	Hybrid	Adzuki bean (male)
Germination habit	epigeal	intermediate	hypogeal
Color of epicotyl	base purple	light green	light green
Petiole length of primary leaves	sessile	short	long
Shape of primary leaves	lanceolate	ovate	broadly cordate
Color of leaves	dark green	dark green	green
Margin of leaflets	entire	often lobed	often lobed
Young pod	hairy	hairy	glabrous

Meiosis in the hybrid

Chromosome pairing at metaphase I in the hybrids was extremely low. The pairing ranged from 0 to 4 pairs with a mean of $2.39_{II} + 17.22_{I}$ and a mode of $2_{II} + 18_{I}$ in 28 cells examined. The maximum and modal pairings are illustrated in Figs. 13 and 14.

Chromosomes in metaphase II were scattered randomly throughout the P.M.C. (Fig. 15) instead of in an orderly arrangement on the two equatorial plates. In telophase, adjacent chromosomes congregated to form microspores at random (Figs. 16 and 17). Examination of 54 sporads showed 66.7% (36) diads (Fig. 18) and 5.6% (3) diads with one or two smaller microspores. Only 7.1% (4) were tetrads while 19.6% (11) were monads or monads with one or two micronuclei.

Extremely low chromosome pairing due to lack of homology, followed by abnormal successive divisions, is considered to be responsible for the hybrid sterility. Because of the sporadic arrangement of the chromosomes instead of orderly congregation on the equatorial plates at second metaphase, sporocytes which form from diads do not seem to be restitution gametes, which would contain whole sets of chromosomes of both genomes of the two species.

4. Crosses between mung bean and rice bean

Crossability

This cross had a much higher success rate when mung bean was used as the female (Table 12). Pod set was 53.1% on mung bean but only 5.6% on rice bean. Seeds were produced only on mung bean.

Figs. 13-18. Meiosis in mung bean (M3) X adzuki bean (A1) hybrids: 13) maximum pairing configuration of $4_{II} + 14_{I}$ (bivalents are indicated by arrows); 14) modal pairing of $2_{II} + 18_{I}$; 15) metaphase II showing 22 chromosomes scattered randomly; 16) congregation of chromosomes into two groups at anaphase II; 17) telophase PMC forming a two-pole cell; 18) three diads. Figs. 13-17 X1,200 and Fig. 18 X480.

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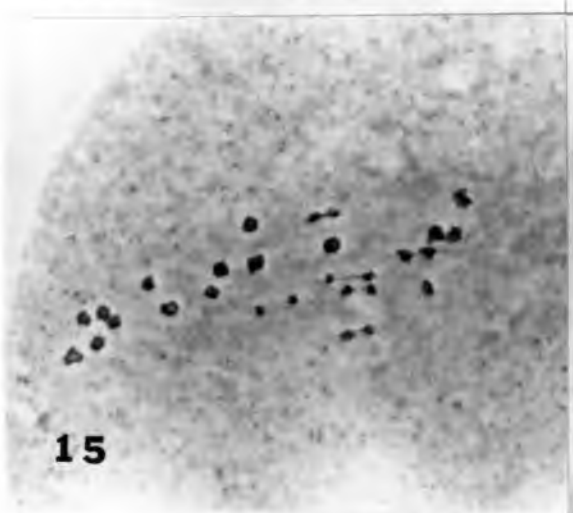

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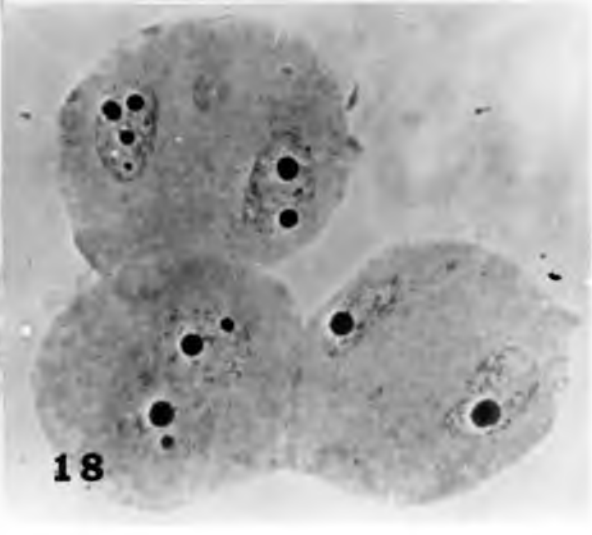

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Table 12. Crossability between mung bean and rice bean

Parents		No. of flowers pollinated	Pod set		No. of pods harvested	Seeds obtained	
Female	Male		No.	%		Empty	Filled
M1	R1	33	16	48.5	15	136	43
	R2	35	22	62.9	17	121	16
Total		68	38	55.9	32	257	59
M2	R1	51	21	41.2	19	97	40
	R2	30	17	56.7	16	120	10
Total		81	38	46.9	35	217	50
M3	R1	33	20	60.6	18	92	44
	R2	31	17	54.8	17	157	14
Total		64	37	57.8	35	249	58
R1	M1	38	1	2.6			
	M2	40	4	10.0			
	M3	30	4	13.3			
Total		108	9	8.3	Pods shed within 3 weeks.		
R2	M1	35	0				
	M2	41	2	4.9			
	M3	31	1	3.2			
Total		107	3	2.8			

X^2- test for pod set and developed seeds

Comparison	Pod set	Developed seeds
M X R vs. R X M	38.43**	
Within M X R	6.00^{ns}	172.34**
between M	1.26^{ns}	26.00**
between R	0.86^{ns}	27.03**
Within R X M	22.08**	
between R	2.75^{ns}	
between M	4.93*	

X^2 value significant at 5% (*) or 1% (**); ns = not significant.

On mung bean, no difference in pod set between parental lines was found. Pods developed normally for 3 weeks, then dried prematurely during the fourth week. The dried pods contained two kinds of seeds, empty and partially filled. The empty seeds did not germinate, but an average of two-thirds of the partially filled seeds germinated. Seed fertility differed between different combinations of parental lines. The number of partially filled seeds obtained per pollination was 0.9 on M1, 0.6 on M2 and 0.9 on M3. This number was 1.1 in pollinations by R1, which is much higher than 0.4 by R2.

When rice bean was used as the female parent, pod set differences between the two rice bean lines were not significant, but the differences in pod set effected by the three mung bean male parents were significant. Pods on rice bean usually shed within 3 weeks after pollination. Embryos older than 10 days were highly disorganized sponge-like bodies.

The reciprocal difference in crossability implies that cytoplasmic factors are responsible for the unidirectional success in this species cross. The isolating mechanism of hybrid inviability results in complete embryo abortion on rice bean and in formation of partially filled or empty seeds on mung bean.

Embryo culture

Since no seeds matured in the rice bean X mung bean crosses, young embryos from these crosses were cultured. Out of 20 11- to 23-day old embryos cultured, none developed into a normal seedling (Table 13). Only one embryo formed a shoot without roots. Deformed

Table 13. Results of culturing hybrid embryos from the cross between rice bean and mung bean

Parents		Age of embryo (days)	State of pod	No. of embryos	Results
Female	Male				
R1	M1	23	dried	1	deformed leaves only
R1	M2	12	green	3	2 callus 1 no growth
		19	wilting	4	1 deformed leaves and upright root with tumored tip, and callus 1 callus 2 no growth
		23	wilting	2	2 callus
R1	M3	11	green	3	2 callus 1 deformed leaves with callus
		17	green	5	1 seedling with no root 4 no growth
R2	M2	21	wilting	2	2 callus

primary leaves developed in two embryos. The extremely rare and abnormal development of embryos in culture is additional evidence of reciprocal differences in the strength of barriers to interspecific gene exchange.

Plant growth and morphology of the hybrids

A characteristic feature of hybrids from mung bean X rice bean was seedling weakness. Hybrid seedlings were very weak and grew very slowly at first (Fig. 19). Abnormal seedlings with three or four primary leaves often appeared. The hybrid plants, however, passed this weak seedling stage and grew very vigorously with thicker stems and larger leaves than the parental species.

The hybrids were intermediate between the two parental species in germination habit, petiole length of the primary leaves, color of epicotyl, color of leaves, days to flower, and flower size. The rice bean characters of indeterminate growth, simple raceme, and glabrous young pods were dominant in the F_1. The edge of the standard was purple and the upper part of the keel was greyish as in mung bean. Trifoliate leaflets are entire in mung bean and often lobed in rice bean, but the hybrids had lobed leaflets only occasionally (Table 14).

The hybrids were multi-branched sub-erect bushes during the winter and vines in the summer. Flowering occurred continuously in the same racemes for an extraordinarily long period (Fig. 20). The hybrids grew as perennial plants, repeatedly produced juvenile branches, and showed no senescence.

Figs. 19-20. The hybrids of mung bean X rice bean: 19) seedlings (M1 X R1); 20) mature plants (M3 X R1).

Figs. 21-23. Meiosis in mung bean X rice bean hybrids: 21) 7_{II} + 8_{I}; 22) 1_{IV} + 3_{II} + 12_{I}; 23) one polyspore of four larger and three smaller microspores. Figs. 21-22 X1,200 and Fig. 23 X480.

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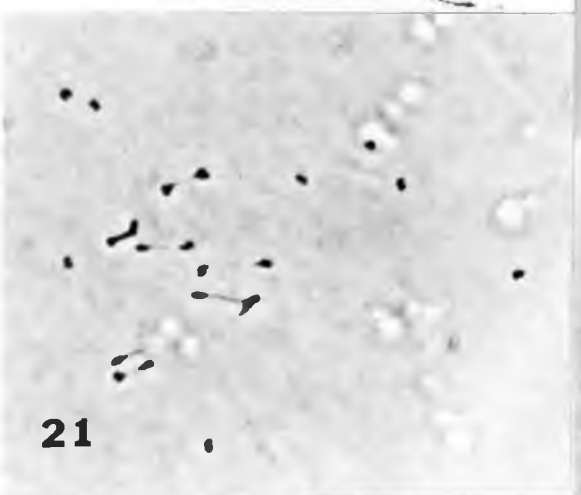

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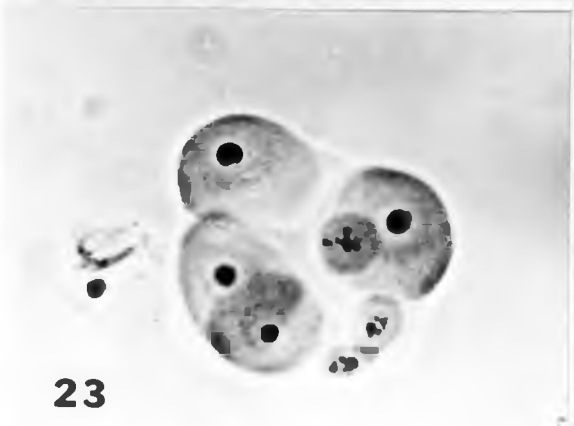

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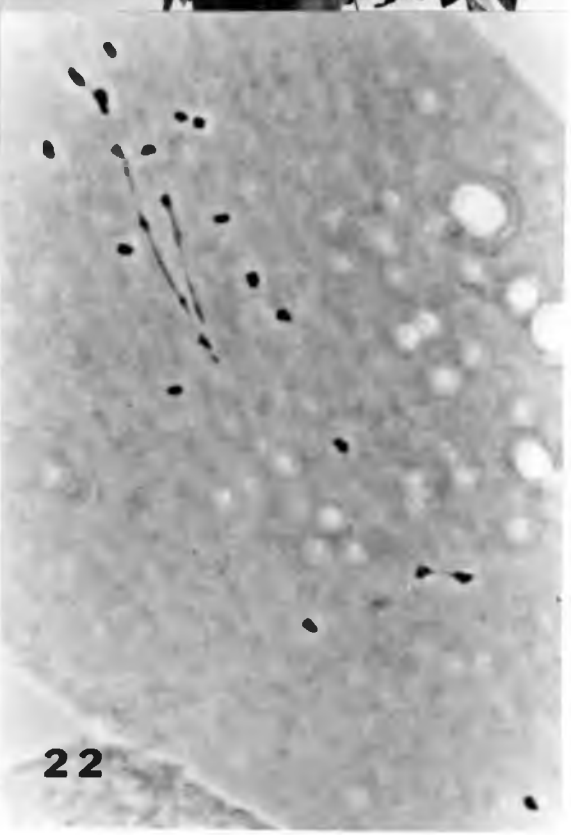

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Table 14. Comparison of mung bean, rice bean, and the hybrids between them

Characters	Mung bean (females)	Hybrids	Rice bean (males)
Germination	epigeal	intermediate	hypogeal
Primary leaves	sessile	short-petioled (2 - 6 mm)	long-petioled (23 - 35 mm)
Color of upper epicotyl	green	light purple	purple
Color of leaves	dark green	intermediate	light green
Margin of leaflets	entire	occasionally lobed	often lobed
Growth habit	determinate	indeterminate	indeterminate
Flowering date (planted on 5/7)	M1: 6/25 M2: 6/12 M3: 6/8	6/23 - 7/14	R1: 9/21 R2: 6/21
Raceme	often compound	simple	simple
Edge of the standard	purple	purple	bright yellow
Width of the standard (mm)	17 - 20	24 - 26	23 - 25
Top of the keel	greyish	greyish	bright yellow
Young pods	hairy	glabrous	glabrous
Per cent stainable pollen	M1: 96.5 M2: 95.7 M3: 91.5	0.8 (M1 X R1) to 5.8 (M3 X R1)	R1: 95.1 R2: 94.4

Fertility of the hybrids

A total of about 150 hybrid plants, grown in both winter and summer, flowered profusely and set a few pods. The young pods, however, dropped in a week and never reached maturity. Pistils of opened flowers had very few or no pollen grains on them. Percentages of stainable pollen were 0.8 in the F_1 of M1 X R1, 0.9 in the F_1 of M1 X R2, 1.6 in the F_1 of M2 X R2, 5.8 in the F_1 of M3 X R1 and 1.7 in the F_1 of M3 X R2 compared to over 90% for the parental species. The pollen grain sizes were highly variable.

Numerous controlled self-pollinations and backcrosses to the parental species in every possible direction failed to produce any seeds.

Complete sterility of the hybrids operates as an absolute reproductive barrier between these two species at the diploid level.

Meiosis in the hybrids

Meiosis in the F_1 plants was highly irregular. In metaphase I, the mean chromosome pairing was $0.09_{IV} + 4.69_{II} + 12.26_{I}$. Maximum pairing was $7_{II} + 8_{I}$ or $1_{IV} + 5_{II} + 8_{I}$ and modal pairing was $5_{II} + 12_{I}$ (Figs. 21 and 22), which are much lower than the maximum 11_{II} and modal $8_{II} + 6_{I}$ reported by Dana (1966). The frequency of the different chromosome configurations observed in the F_1 hybrids is presented in Table 15.

The quadrivalents observed during meiosis, as Dana (1966) pointed out, indicate that at least one reciprocal translocation has occurred during chromosomal differentiation of these two species.

The lack of congregation of the bivalents and random distribution of the univalents resulted in unequal separation of chromosomes at

Table 15. Meiotic analysis of hybrids of mung bean X rice bean

Hybrids	No. of cells examined		Chromosome configurations		
			IV	II	I
		10		6	10
		9		5	12
M1 X R1		7		4	14
		4		3	16
	Total	30	Average	4.83	12.33
		2		7	8
		3		6	10
		1	1	3	12
		7		5	12
M1 x R2		2	1	2	14
		3		4	14
		3		3	16
	Total	21	Average 0.14	4.52	12.38
		1		7	8
		2		6	10
M2 X R1		3		5	12
		1		4	14
		1		3	16
	Total	8	Average	5.13	11.75
		2		6	10
		1	1	3	12
		3		5	12
M2 X R2		1	1	2	14
		9		4	14
		4		3	16
	Total	20	Average 0.10	4.00	13.60
		1	1	5	8
		8		7	8
		4		6	10
M3 X R1		1	1	3	12
		9		5	12
		1		4	14
		1		3	16
	Total	25	Average 0.08	5.60	10.48
		1	1	5	8
		2	1	4	10
		1		6	10
M3 X R2		1	1	2	14
		7		4	14
		5		3	16
	Total	20	0.20	4.05	13.10
	Grand total	124	Grand average 0.09	4.69	12.26

anaphase I. The second division was also abnormal. Chromosomes in metaphase II failed to orient properly, which resulted in random distribution of the second anaphasic chromosomes.

Polyspory was another peculiarity in microsporogenesis in these hybrids, as was also reported by Sawa (1975). The number of microspores formed from a microsporocyte was highly variable. Monads, diads, and triads alone or together with up to six extra micronuclei were observed at varying frequencies (Fig. 23).

Considering the extremely irregular chromosome associations in both meiotic divisions and the polyspory observed, the production of a gamete with a complete chromosome set of one genome would hardly be expected. Therefore, the complete sterility of the hybrids is apparently due to the lack of chromosomal homology and resultant meiotic irregularities, as Dana (1966) already pointed out.

Amphidiploid

Thirty vigorous cuttings were taken from the completely sterile F_1 plants and treated with colchicine. The colchicine-treated cuttings rooted well and grew vigorously when transplanted to plastic pots. Of these 30 plants, 17 (56.6%) produced fertile branches which yielded ripe pods with crinkled seeds. When the crinkled seeds were soaked in water, most appeared normal and germinated. Some of the seeds did not absorb water readily and germination was delayed.

The seedlings grew virorously with no seedling weakness as in the original diploid hybrids (Fig. 24). The general morphological characters and plant growth were very similar to the diploid hybrids.

Figs. 24-25. Mung bean (M2) X rice bean (R2) amphidiploid (C_2) plants: 24) seedlings; 25) mature plants.

Figs. 26-29. Meiosis in the mung bean X rice bean amphidiploids: 26) diakinesis with 22 bivalents (arrow indicates 2 bivalents superimposed in the picture); 27) metaphase with 22 bivalents; 28) metaphase II showing equal separation of chromosomes; 29) telophase showing equal distribution of chromosomes into four microspores. Figs. 26 and 29 X480 and Figs. 27 and 28 X1,200.

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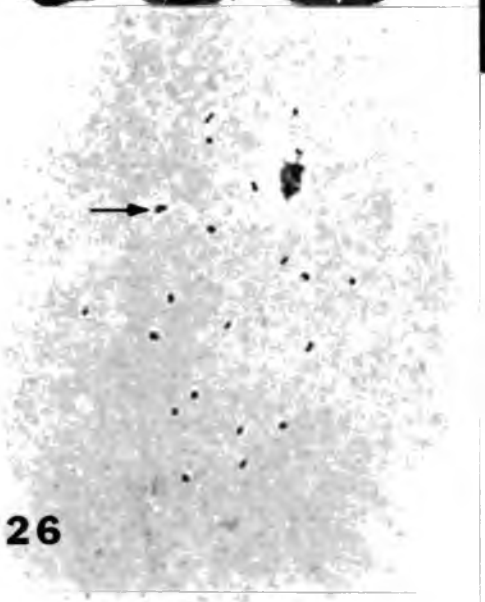

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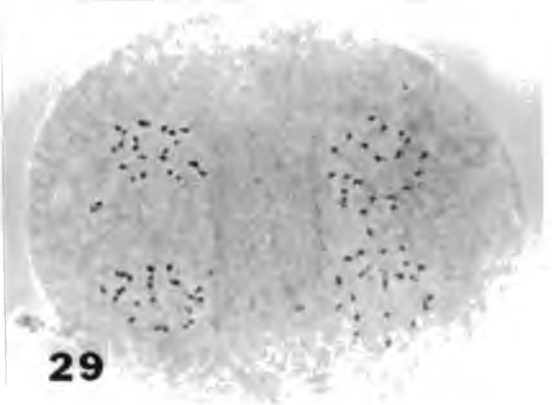

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The plants grew very profusely as multi-branched semi-erect bushes during the winter (Fig. 25) but as vines during the summer. They began to flower about the same time as the diploid hybrids and were perennial.

The gigas effect of polyploidy was observed in the floral characters. The width of the standard increased to 27 - 30 mm from 24 - 27 mm in the F_1 hybrids. The flower color was darker yellow and the petals were thicker.

Pollen stainability increased to as much as 75.5 to 83.6% and seed number per pod averaged 4.2. Pod set was very low at first, but increased later. The seeds were crinkled like the ones produced by the colchicine-treated plants (C_1). Numerous crosses with the parental species and the F_1 hybrids failed to yield any pods.

Meiosis in the amphidiploids showed 22 bivalents (Figs. 26-27) and orderly disjunction in both anaphases (Figs. 28-29), similar to that reported by Sawa (1975). Amphidiploid progenies up to C_4 generation were grown. Plants with plump seeds or increased pod set were not observed.

The complete sterility of the hybrids of mung bean X rice bean can be effectively overcome by chromosome doubling, indicating that sterility in F_1 diploids is due largely to chromosomal factors. The crinkled seeds and low pod set may mean that some adverse genic interactions exist and thus these amphidiploids are unlikely to become an economic crop without further extensive breeding work. However, their exceptionally vigorous vegetative growth with strong perennialism and substantial seed production may be useful for a soil-building or forage crop.

5. Crosses between black gram and mung bean

Crossability

This cross was successful only when mung bean was used as the female parent. Pod set was 29.4% on black gram and 58.6% on mung bean, only slightly lower than for intraspecific pollinations. Therefore, it seems that there are no barriers to pod set between these two species. The results of the crosses are presented in Table 16.

On black gram, pod set varied according to the combinations of parental lines used. The B1 X M2 cross produced the highest pod set (46.0%) among six cross combinations. Pods seemed to develop normally but dried prematurely. Dried pods contained two kinds of seeds: empty and slightly filled seeds. Neither kind of seeds germinated. Some embryos appeared to be fairly normal, but the radicle and growing point were poorly developed and the cotyledons were distorted and fluffy.

On mung bean, differences in pod and seed set were highly significant between mung bean lines. M2 had the lowest pod set (43.3%) as well as the lowest seed number per pod (6.1) among the three lines. No effect of black gram pollen on pod set was noticed. The pods developed normally up to maturity. Ripe pods contained partially filled seeds, which appeared normal when soaked in water and germinated well.

There seem to be no barriers to fertilization between these two species, at least in some cross combinations of lines of the two species. However, there are barriers to seed development which result

Table 16. Crossability between black gram and mung bean

Parents		No. of flowers crossed	Pods harvested		Seeds obtained	
Female	Male		No.	%	Empty	Filled
B1	M1	36	8	22.2	13	11
	M2	37	17	45.9	31	19
	M3	33	8	24.2	12	14
Total		106	33	31.1	56	44
B2	M1	30	11	36.7	37	8
	M2	35	6	17.1	7	8
	M3	30	9	30.0	7	12
Total		95	26	27.4	51	28
M1	B1	32	23	71.9		191
	B2	30	21	70.0		171
Total		62	44	71.0		362
M2	B1	32	13	40.6		83
	B2	28	13	46.4		75
Total		60	26	43.3		158
M3	B1	33	22	66.7		207
	B2	32	17	53.1		173
Total		65	39	60.0		380

X^2- test for pod set and developed seeds

Comparison	Pods harvested	Filled seeds obtained
B X M vs. M X B	9.56**	383.75**
Within B X M	18.92**	156.47**
between B	0.24^{ns}	0.007^{ns}
between M	0.43^{ns}	19.65**
Within M X B	14.99**	295.07**
between M	6.67**	143.85**
between B	0.08^{ns}	0.94^{ns}

**significant at 1% level; ns = not significant.

in empty and nonviable seeds on black gram and slightly defective but viable seeds on mung bean.

Embryo culture

Fourteen hybrid embryos on black gram were cultured. None of these embryos developed into normal seedlings. As shown in Table 17, three embryos formed callus only and others did not show any sign of growth. Thus, incompatibility between the degenerating embryos and the developing endosperm does not seem to be the cause of failure of this cross.

The reciprocal difference in crossability between black gram and mung bean suggests that a disharmonious interaction between genic and cytoplasmic factors may be responsible for the embryo degeneration when the cross is made on black gram.

Plant growth and morphology of the hybrids

There was a difference in the vitality of hybrids from different lines of mung bean. The hybrids displayed hybrid vigor in their early seedling growth (Fig. 30). Vigorous plant growth extended throughout the growing season in the hybrids of M1 and M2. However, the hybrids from crosses with M3 as females (M3 X B1 and M3 X B2) suddenly lost vigor upon forming flower buds and barely grew from then on.

The hybrids resembled mung bean in having a purple-tinged edge of the standard and grey color in the upper part of the keel; resembled black gram in having a simple raceme, long-hairy and upright pods, ovoid seeds, and a concave hilum cushion; and were intermediate in foliage color (Table 18).

Table 17. Results of culturing hybrid embryos from the cross between black gram and mung bean

Parents		Age of embryo (days)	State of pod	No. of embryos	Results
Female	Male				
B1	M2	11	green	1	no growth
		16	green	1	no growth
B1	M3	11	green	3	3 callus
		14	green	1	no growth
B2	M2	16	green	4	no growth
		35	dried	1	no growth
B2	M3	13	green	3	no growth

Figs. 30-31. The hybrids of mung bean (M2) X black gram (B2): 30) seedlings; 31) mature plants.

Figs. 32-35. Meiosis in mung bean X black gram hybrids: 32) metaphase I showing 11 bivalents; 33) diakinesis - $1_{IV} + 8_{II} + 2_{I}$ (univalents are indicated by arrows); 34) diakinesis - $1_{IV} + 9_{II}$; 35) one polyspore of five microspores. Figs. 32-34 X1,200 and Fig. 35 X480.

Fig. 36. A BC_1 plant from mung bean X (mung bean X black gram) (M2 X F_1 of M2 X B2), showing increased pod set.


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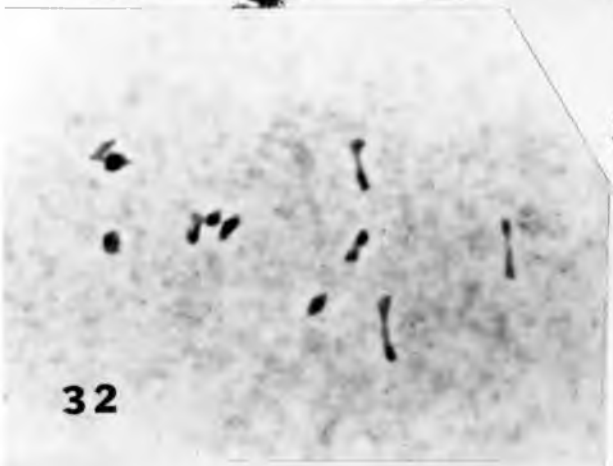
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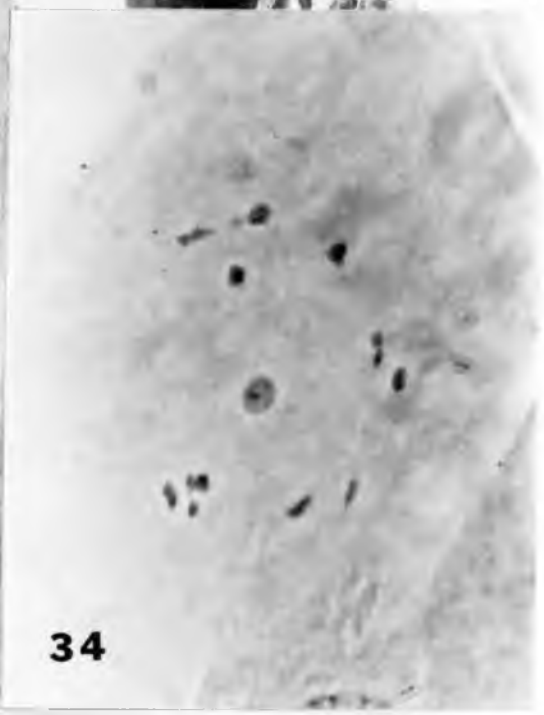
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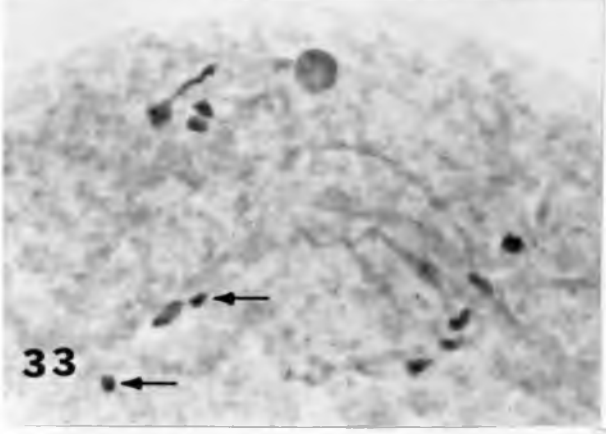
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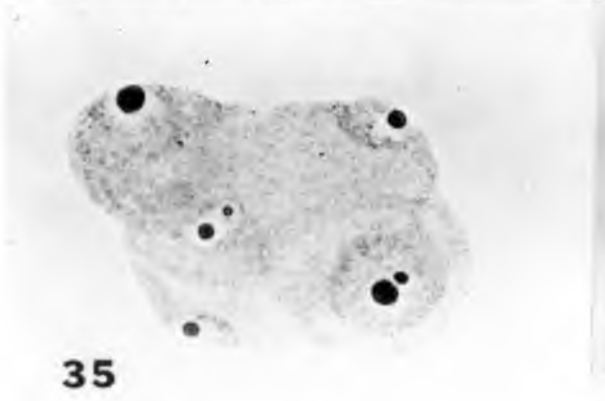
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Table 18. Morphological comparison of mung bean, black gram, and the hybrids between them

Characters	Mung bean (females)	Hybrids	Black gram (males)
Color of leaves	dark green	intermediate	light green
Raceme	often compound	simple	simple
Edge of the standard	purplish (M2, M3)	purplish	dark yellow
Top of the keel	greyish	greyish	dark yellow
Hairs on pod	short	long	long
Pods at maturity	radiate or drooping	upright	upright
Shape of seed	globose	ovoid	ovoid
Color of seed	green	brown and black spotted	dark grey and white spotted
Hilum cushion	not concave	concave	concave
Per cent stained pollen	96.5 (M1) 95.7 (M2) 91.5 (M3)	24.0 (M3 X B2) to 26.6 (M1 X B2)	95.9 (B1) 95.1 (B2)

Plant growth was indeterminate like black gram; multi-branched with slight tendency to vine in the winter and viny during the summer. The F_1 hybrids of M3 X black gram also had indeterminate growth, but did not become viny in the summer because of the severely weakened plant growth, and died after a short period of fruiting.

Fertility of the hybrids

The hybrids were partially fertile. The hybrid plants flowered profusely but pod set was very low (Fig. 31). A few ripe pods harvested from open-pollinated flowers contained usually one or rarely two seeds. The seeds were of various kinds, from empty to fully developed. Some seeds had burst seed coats. Pollen stainability ranged from 24.0% to 26.6%. The stained pollen grains were comparatively uniform, but the unstained grains were much smaller and highly variable in size.

Viable seeds were produced when the hybrids were used as both male and female parents in backcrosses to the parental species.

Meiosis in the hybrids

Many kinds of irregularities were observed during microsporogenesis in the hybrids. Chromosome configurations at diakinesis and at metaphase I are presented in Table 19. Complete bivalent formation occurred in 28.6% of 147 cells examined (Fig. 32). The other cells contained one quadrivalent and/or two or four univalents (Figs. 32 and 34). The mean chromosome configuration was $0.37_{IV} + 9.59_{II} + 1.34_{I}$. Often the bivalents failed to orient properly on the equatorial plate. Heteromorphic pairing was noticed in diakinesis. Chromosome

Table 19. Meiotic analysis of hybrids of mung bean X black gram

Hybrids	No. of cells examined		Chromosome configurations			
			IV	II	I	
M1 X B1		5	1	9		
		7		11		
		2	1	8	2	
		5		10	2	
		1	1	7	4	
		5		9	4	
	Total	25	Average	0.32	9.60	1.52
M1 X B2		1	1	9		
		4		11		
		2	1	8	2	
		10		10	2	
		1	1	7	4	
		6		9	4	
	Total	24	Average	0.17	9.58	2.16
M2 X B1		11	1	9		
		13		11		
		1	1	8	2	
		1		10	2	
	Total	26	Average	0.46	10.00	0.16
M2 X B2		4	1	9		
		7		11		
		6	1	8	2	
		7		10	2	
		1	1	7	4	
		2		9	4	
	Total	27	Average	0.41	9.48	1.40
M3 X B1		5	1	9		
		6		11		
		7	1	8	2	
		6		10	2	
		1	1	7	4	
		2		9	4	
	Total	27	Average	0.48	9.33	1.41
M3 X B2		4	1	9		
		5		11		
		2	1	8	2	
		3		10	2	
		1	1	7	4	
		3		9	4	
	Total	18	Average	0.39	9.50	1.44
	Grand Total	147	Grand Average	0.37	9.59	1.34

bridges occurred in 3 cells and 1 to 5 laggards were observed in 5 cells out of 29 cells examined at anaphase I. Chromosome distribution at anaphase I was studied in 21 cells. Chromosomes had separated equally 11-11 in 11 cells, 10-12 in 9 cells, and 13-9 in one cell. Polyspory was observed in many tetrads (Fig. 35).

The results show that these two species are closely related with chromosome differentiation having occurred through one major reciprocal translocation. This confirms the reports of Dana (1966a), and De and Krishnan (1966b). The low pod set compared with the fairly high frequency of complete bivalent formation indicates that the cause of the sterility may be partly due to either chromosomal heterozygosity or genic interaction. From pachytene chromosome analysis, De and Krishnan (1966) reported that a terminal deletion and an interstitial duplication are also involved.

The F_2 and backcross generations

Germination of well developed seeds from F_1 plants was highly irregular. Seeds germinated sporadically over an extended period. Some seeds remained hard and germinated late and others failed to germinate at all. Out of 74 seeds, 47 (60%) germinated in 10 days (Table 20). Germination in 3 days was quite uniform in mung bean, black gram and the F_1.

Seedling lethality and weakness were other characteristics found in the F_2 generation. Fifteen seedlings died during early or late seedling stages, among which three were complete albinos, four were sectorial albinos, and the rest died after forming clusters of curly leaves or when flower buds formed. The hybrid vigor of the F_1 was

Table 20. The F_2 generation of mung bean X black gram crosses

Crosses	No. of seeds sown	Germi-nated seeds	No. of plants flowered	% stained pollen	No. of plants set pods
M1 X B1	13	9	7	9.8-51.5	1
M1 X B2	13	7	5	12.1-54.3	2
M2 X B1	13	8	6	16.1-71.6	5
M2 X B2	13	8	5	11.9-68.6	3
M3 X B1	12	8	5	8.3-69.5	4
M3 X B2	10	7	4	17.0-57.6	3
Total	74	47	32	8.3-71.6	18

completely lost in the F_2 generation. No individual F_2 plant grew as vigorously as the F_1 plants.

Wide variability in fertility was observed in the F_2 generation. Pollen stainability ranged from 9.26 to 71.63% (Table 20). Eighteen out of 32 plants which flowered produced pods with well-developed seeds.

All combinations of backcrosses to the parental species produced viable seeds (Table 21). No differences in pod set between the backcrosses to mung bean and black gram were found. However, differences due to the direction of the crosses to each parental species were highly significant. In backcrosses to black gram, pod set and seed production were much higher when the F_1 plants were used as the females. Pod set was significantly higher on F_1 plants in backcrosses to mung bean, but there was no difference in seed number, because of the higher seed number in the mung bean pod.

Hybrid lethality was much lower in the backcross progenies (BC_1) than in the F_2. Out of 38 seeds, 27 (70%) seedlings were obtained, of which 3 died during seedling stages, 3 did not flower, and 21 flowered. All of the BC_1 plants showed a mixture of mung bean and black gram characters. Plant growth was highly diverse, from dwarf to normal growth, and from bush to vine. Fertility varied in the backcross progenies. Pollen stainability ranged from as low as 4.0% to as high as 75.1%. Three plants with less than 20% stainable pollen did not produce any mature pods. Pot set was very high in some plants (Fig. 36).

Table 21. Backcrosses of hybrids of mung bean X black gram to their parents

Crosses		No. of flowers crossed	Pods harvested		No. of seeds obtained	Progenies grown	
Female	Male		No.	%		No.	% stained pollen
M1B1	M1	14	2	14.3	2	1	27.5
(F_1)	B1	11	2	18.2	4	2	no flower
M1	M1B1	10	1	10.0	2	2	38.6, 67.4
B1	(F_1)	10	1	10.0	1	1	41.9
M1B2	M1	11	0				
(F_1)	B2	13	1	7.7	1	1	25.1
M1	M1B2	10	0				
B2	(F_1)	15	1	6.7	1	1	no flower
M2B1	M2	16	5	31.3	5	3	30.3-63.6
(F_1)	B1	17	7	41.2	5	3	33.0-75.1
M2	M2B1	12	0				
B1	(F_1)	10	0				
M2B2	M2	13	1	7.7	1	0	
(F_1)	B2	13	2	15.4	1	0	
M2	M2B2	12	1	8.3	4	3	6.36-66.0
B2	(F_1)	10	0				
M3B1	M3	16	3	18.8	1	1	50.3
(F_1)	B1	17	6	35.3	3	2	30.0, 54.3
M3	M3B1	15	1	6.7	5	3	15.9-64.8
B1	(F_1)	10	0				
M3B2	M3	8	0				
(F_1)	B2	5	2	40.0	2	1	4.0
M3	M3B2	7	0				
B2	(F_1)	2	0				

X^2- test for pod and seed set

Comparison	Pods	Seeds
BCM vs. BCB	1.77^{ns}	0.005^{ns}
F_1 X B vs. B X F_1	17.44**	12.53**
F_1 X M vs. M X F_1	4.89*	0.93^{ns}

X^2 value significant at 5% (*) or 1% (**); ns = not significant.

Germplasm exchange between mung bean and black gram is highly possible through backcrosses to either parent.

6. Crosses between adzuki bean and rice bean

Crossability

When adzuki bean was used as the female, there was no significant difference in pod set between the two lines of adzuki bean (Table 22). The difference between the two rice bean lines used as pollen parents was also not significant. The overall pod set in these two crosses was 55.6% which is significantly higher than the 23.0% pod set for intraspecific crosses on adzuki bean. Pods developed normally and reached their maximum size in 3 weeks, when they suddenly discolored, lost turgidity, and collapsed. Pods older than one week contained only highly distorted, sponge-like embryos.

When rice bean was used as the female, significant differences were found between parental lines of both species. R2 set more pods (31.3%) than R1 (10.8%) and A2 was a better pollinator (27.3%) than A1 (12.7%). The highest pod set (36.6%) was from the cross R2 X A2. Pods developed slowly and were shed 2 to 3 weeks after pollination. Embryos from pods 11 days old or more appeared normal and healthy. Shrivelled seeds obtained from dried pods appeared normal when soaked in water but failed to germinate.

Apparently, there is no barrier to fertilization in adzuki bean X rice bean crosses. The higher pod set on adzuki bean in the crosses with rice bean is most likely due to scant pollen production on adzuki bean during the winter. However, in rice bean X adzuki bean crosses,

Table 22. Crossability between adzuki bean and rice bean

Parents		No. of flowers crossed	No. of pods set	% pod set
Female	Male			
A1	R1	33	16	48.5
	R2	32	20	62.5
Total		65	36	55.4
A2	R1	32	20	62.5
	R2	38	19	50.0
Total		70	39	55.7
R1	A1	29	1	3.4
	A2	36	6	16.7
Total		65	7	10.8
R2	A1	26	6	23.1
	A2	41	15	36.6
Total		67	21	31.3

X^2- test for pod set

Comparison	X^2 value
A X R vs. R X A	15.37**
Within A X R	3.17^{ns}
between A	0.001^{ns}
between R	0.001^{ns}
Within R X A	28.56**
between R	10.05**
between A	5.29*

X^2 value significant at 5% (*) or 1% (**); ns = not significant.

the differential pod set on R1 and R2 indicates that barriers to fertilization vary according to rice bean lines used in the crosses.

Extremely early degeneration of embryos when adzuki bean was used as the female was another directional difference. The presence of normal pod development suggests that growth substances promoting fruit development were present after degeneration of the embryos.

Embryo abortion and failure of partially filled seeds to germinate are effective barriers to gene exchange between adzuki bean and rice bean.

Embryo culture

Of 17 embryos 8 to 11 days old cultured from adzuki bean X rice bean crosses, none developed into normal viable seedlings (Table 23). One embryo developed abnormal primary leaves with callus, and three others callused only. The remainder of the embryos failed to exhibit any sign of growth.

Forty seven embryos 11 to 24 days old from rice bean X adzuki bean crosses were cultured. As shown in Table 23, viable seedlings were obtained only from the cross R2 X A2. Twelve pale green and 12 healthy seedlings developed out of 33 embryos cultured. All the pale green seedlings and five of the healthy seedlings died during seedling stages, while the other seven healthy seedlings reached maturity.

The exact cause of the seedling etiolation is uncertain, but it is evident that normal, viable hybrids can be secured in certain rice bean X adzuki bean crosses by embryo culture.

The reciprocal difference in hybrid embryo development in these crosses implies that cytoplasmic differentiation is a principal factor

Table 23. Results of culturing hybrid embryos from crosses between adzuki bean and rice bean

Parents		Age of embryo (days)	State of pod	No. of embryos	Results
Female	Male				
A1	R1	18	green	1	no growth
A1	R2	13	green	1	no growth
A2	R1	8	green	3	no growth
		10	green	3	1 callus 2 no growth
		16	green	3	no growth
A2	R2	10	green	2	no growth
		14	green	2	1 callus 1 no growth
		15	green	2	1 deformed leaves with callus 1 no growth
R1	A1	15	wilting	3	no growth
R1	A2	11	wilting	2	no growth
		16	wilting	4	4 etiolated seedlings
R2	A1	14	dropped	3	1 etiolated seedling 2 no growth
		22	green	1	etiolated seedling
R2	A2	12	green	5	2 mature hybrids 3 seedlings died
		18	green	5	1 mature hybrid 4 seedlings died
		20	green	4	4 etiolated seedlings
		21	green	3	3 etiolated seedlings
		21	wilting	10	8 etiolated seedlings 2 no growth
		21	dropped	1	1 mature hybrid
		22	green	2	2 mature hybrids
		24	green	1	1 mature hybrid
		24	dried	2	no growth

involved in the evolution of isolating mechanisms between these two species.

Morphology of the hybrid

The shape of the primary leaves of the hybrid was ovate (Fig. 37) as compared to linear lanceolate in rice bean and broadly cordate in adzuki bean (Table 24). The hybrids resembled the female species, rice bean (R2), in many ways. The rice bean characters of elongated raceme, blackish-grey color of the ripe pod, dark yellow flower color, greenish-yellow seed color, and concave hilum cushion appeared in the hybrids. The constriction of the pod between the seeds and the shape of the seeds were intermediate between the two parents. The green foliage color and erect type of growth of the adzuki bean were dominant (Fig. 38). Trifoliate leaflets were lanceolate in the hybrids, but rhombic in both parents. The hybrids produced much heavier seeds than either adzuki bean or rice bean. The length of pod and number of seeds per pod were reduced in the hybrids. The reduction of pod length and seed number is perhaps the result of reduced fertility in the inter-specific hybrid.

Fertility of the hybrid

The hybrids produced 76.0% stainable pollen as compared to 94.4% in R2 and 91.0% in A2. The number of seeds per pod was 3.2 which is 52.5% of the midparent value (6.0). Viable seeds were produced in backcrosses to both parental species in all combinations.

It seems that once embryo abortion is circumvented by embryo culture no other reproductive isolation barrier exists to restrict gene exchange between these two species.

Figs. 37-38. The hybrids of rice bean (R2) X adzuki bean (A2) obtained by embryo culture: 37) seedlings; 38) mature plants.

Figs. 39-41. Regular meiosis in rice bean (R2) X adzuki bean (A2) hybrid: 39) metaphase I - 11_{II}; 40) metaphase II; 41) telophase. All figures X1,200.

Figs. 42-43. F_2 plants of rice bean (R2) X adzuki bean (A2): 42) seedlings showing segregation for the shape of primary leaves; 43 mature plants with good pod set.

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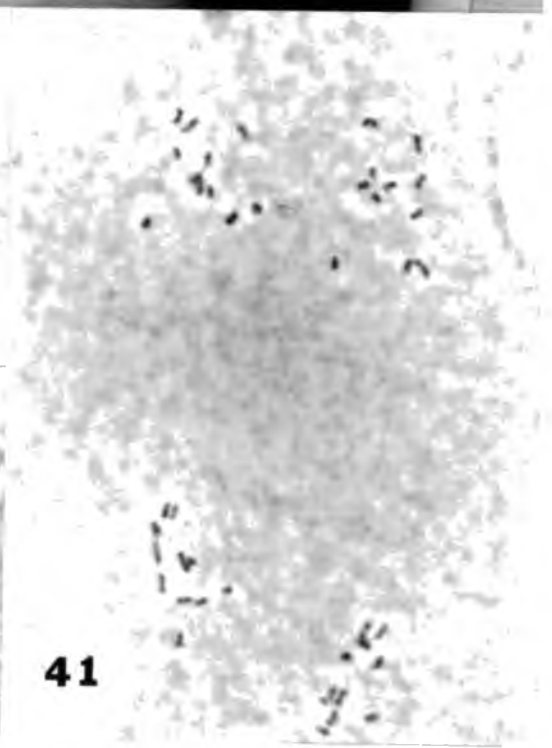

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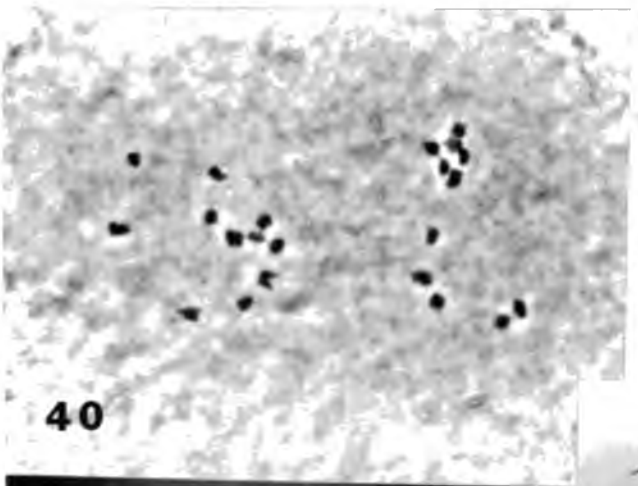

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Table 24. Comparison of rice bean (R2), adzuki bean (A), and the hybrid between them

Characters	Rice bean (female)	Hybrid	Adzuki bean (male)
Shape of primary leaves	linear lanceolate	ovate	broadly cordate
Color of epicotyl	purple	light purple	light green
Color of leaves	light green	green	green
Shape of leaflets	rhombic	lanceolate	rhombic
Constriction of pod	smooth	intermediate	conspicuous
Plant growth	sub-erect, multi-branched	erect, simple stem	erect, simple stem
Raceme	elongated	elongated	compact
Color of ripe pod	blackish grey	blackish grey	straw
Seed color	greenish yellow	greenish yellow	maroon
Hilum cushion	concave	concave	not concave
Seed size (mm) length X width	7.1 X 4.1	6.9 X 4.5	5.5 X 4.2
Seed weight (mg)	7.5	9.0	6.6
Pod length (cm)	8.4	6.1	7.5
No. of seeds per pod	6.3	3.2	5.7
Pollen stainability (%)	94.4	76.0	91.0
Flower color	bright yellow	bright yellow	light yellow

Meiosis in the hybrid

The hybrids regularly formed 11 bivalents at first metaphase in 26 cells examined (Fig. 39). As in the parental species, precocious separation of a few bivalents was observed. Otherwise the separation and movement of the chromosomes in both anaphases was normal (Figs. 40 and 41).

The F_2 and backcross generations

The seeds from F_1 plants germinated normally. In 23 F_2 plants, continuous variation was observed in the shape of the primary leaves (Fig. 42) and epicotyl color, indicating multiple genes are involved in the inheritance of these characters. Plant growth was normal except for two dwarfs. The dwarf plants grew slowly and took exceptionally long to produce a few flowers. Five plants had lanceolate leaflets as in the F_1 and the remaining 16 plants had the rhombic leaflets of both parental species. The growth habit was an erect bush with a simple main stem in 10 plants and sub-erect with multiple stems in 11 plants. The red seed color of the adzuki bean was recovered in four plants. Segregation for fertility was observed. Stainable pollen ranged from 48.5 to 93.3% in five plants examined. Plants with a high percentage of stainable pollen also had good pod set (Fig. 43).

In backcrosses to the parental species, viable seeds were produced in all of the four possible combinations. Because of insufficient flowering of A2 at the right time, A1 and the inter-line hybrid (A2 X A1) were sometimes substituted for A2 in these backcrosses.

As shown in Table 25, reciprocal differences in the backcrosses to adzuki bean are not significant, but reciprocal differences are highly

significant in the backcrosses to rice bean. The extremely low pod set on rice bean (8.3%) was probably caused by red spider mite damage during fruit development. Rice bean was severely damaged by the mites long before adzuki bean and the F_1 hybrids showed visual injury. A lower rate of viable pollen in F_1 plants probably caused lower pod set on rice bean also. The differences between the backcrosses to the paternal and maternal species indicate that cytoplasmic factors may be involved in seed failure in this species cross.

Progenies from all of the possible backcrosses were produced. However, two plants from the A2 X F_1 cross and one plant from the (A2 X A1) X F_1 cross died before flowering. The remaining plants reached maturity and produced normal seeds. Seed production of the backcross progenies was directly proportional to the percentages of their pollen stainability (Table 25). The adzuki bean seed color, which was recessive in the F_1, was recovered in three out of five backcrosses to adzuki bean. The plant characters of the backcrosses resembled the parental species used in the cross, but plants with all the characters of the backcross parental species did not appear.

This species hybridization indicates that embryo abortion due to disharmonious interaction between cytoplasmic factors of one species and genic factors of the other can be overcome only by culturing developing embryos from the cross with rice bean as the female parent. Success also seems to depend upon the particular combination of genotypes of the two species. The high fertility of the F_1, F_2 and backcross generations indicates that no barrier other than embryo abortion blocks the exchange of genes between these two species. Therefore, it

Table 25. Backcrosses of the rice bean (R2) X adzuki bean (A) hybrid to the parental species

Crosses		No. of flowers crossed	Pods harvested		No. of seeds obtained	No. of progenies flowered	% stained pollen
Female	Male		No.	%			
R2A2 F_1	A2	5	2	40.0	1	1	50.1
	A1	4	1	25.0	4	2	75.1 80.3
	A2A1	10	1	10.0	1	0	
Total		19	4	21.1	6	3	68.7
A2	R2A2 F_1	6	3	50.0	5	0	
A2A1		15	3	20.0	3	2	88.7 70.1
Total		21	6	28.6	8	2	79.4
R2A2 F_1	R2	18	15	83.3	87	11	75.1 to 93.3
R2	R2A2 F_1	24	2	8.3	3	1	88.3

X^2- test for pod and seed set

Comparison	Pods	Seeds
BCA vs. BCR	3.66^{ns}	128.95**
F_1 X A vs. A X F_1	1.14^{ns}	0.61^{ns}
F_1 X R vs. R X F_1	61.37**	447.10**

**highly significant; ns = not significant.

appears that these two species are very closely related and that an isolation mechanism has evolved primarily through cytoplasmic differentiation resulting in embryo abortion. Accordingly, once embryo abortion is overcome, it would be possible to exchange germplasm between these two species and thus improve either adzuki bean or rice bean.

GENERAL DISCUSSION AND CONCLUSIONS

A variety of isolating mechanisms were found to operate between Vigna radiata, V. umbellata, V. angularis and V. mungo. The results of the hybridizations among these species are summarized in Table 26. Normal pod set means pod set in at least one cross was equal to that found in intraspecific pollinations. After pods had set, in most cases the embryo aborted before reaching maturity. Only in the pollinations of mung bean by black gram and rice bean was there not complete embryo abortion. Immature embryos were cultured on artificial medium in all pollinations where the embryos did not mature naturally. In some cases, successful embryo culture was followed by loss of all seedlings in the seedling stage. A further difference was noted in that some seedlings which survived remained weak, while others grew well. Finally, some hybrids were completely sterile, while others were only partially sterile. These differences and the meiotic configurations found in the hybrids are shown in Table 26.

Two or three lines of each species were used in the crosses. The variability in crossability shown by different lines of the same species suggests that the use of more than one genotype as well as intraspecific hybrids is desirable when making interspecific crosses.

Reciprocal differences in crossability were found in all interspecific crosses. The differences were observed in pod set, embryo abortion, embryo culture, and seedling lethality.

The results of the six species crosses are summarized as follows:

The cross between adzuki bean and black gram was the least successful. Although pod set was normal when adzuki bean was used as the

Table 26. Summary of hybridizations among the four Vigna species

Crosses		Pod	Embryo	Embryo	Seedling	Hybrid	Hybrid	Hybrid
Female	Male	set	abortion	culture	lethality	weakness	sterility	meiosis
Adzuki bean X Black gram		normal	complete	partial	complete			
Black gram X Adzuki bean		partial	complete	partial	complete			
Black gram X Rice bean		normal	complete	partial	partial	complete		
Rice bean X Black gram		partial	complete	none				
Adzuki bean X Mung bean		normal	complete	partial	complete			
Mung bean X Adzuki bean		partial	complete	partial	partial	none	complete	2.39II+17.22I
Mung bean X Rice bean		normal	partial		partial	none	complete	0.09IV+4.69II +12.26I
Rice bean X Mung bean		partial	complete	none				
Black gram X Mung bean		normal	complete	none				
Mung bean X Black gram		normal	none		none	partial	partial	0.37IV+9.59II +1.34I
Adzuki bean X Rice bean		normal	complete	none				
Rice bean X Adzuki bean		normal	complete	partial	partial	partial	partial	11II

female, embryo abortion was complete and no seedlings obtained from embryo culture survived beyond the seedling stage. These two species appear to be the most distantly related of the four species.

The cross between black gram and rice bean was also very difficult. Some seedlings obtained by embryo culture from crosses on black gram reached adult stage, but died before flowering. Therefore, these species are also considered to be very distantly related.

The cross between adzuki bean and mung bean was somewhat more successful. Hybrids from mung bean produced by embryo culture flowered profusely but were completely sterile. Lack of chromosome pairing, shown by an average of $2.39_{II} + 17.22_{I}$, seems likely to be a principal cause of sterility. These two species also seem to be distnatly related.

The cross between mung bean and rice bean was somewhat easier than the last. Embryo abortion is not complete so that some crinkled but viable seeds are produced on mung bean. However, the hybrids, with average chromosome pairing of $0.09_{IV} + 4.69_{II} + 12.26_{I}$, are still completely sterile. Colchicine-induced amphidiploids showed regular meiosis with 22 bivalents and greatly improved fertility, indicating that at least in this combination much of the sterility of the diploid hybrid is caused by chromosomal factors. However, low pod set and defective seeds in the amphidiploid indicate that some adverse genic interactions are still found. These two species must be more closely related than any of the previous combinations.

Black gram and mung bean seem to be very closely related. Hybrid seed development, when mung bean was used as the female, was almost

normal and germination was good. The F_1 hybrids were partially fertile. One quadrivalent in 37% of the cells examined indicates that one reciprocal translocation has occurred during chromosomal differentiation of these two species. Segregation for morphological characters and the appearance of highly fertile individuals in the F_2 and BC_1 demonstrate the possibility of gene exchange between these two species.

Adzuki bean and rice bean also seem to be closely related. Hybrids obtained by culture of embryos from rice bean were highly fertile with completely normal bivalent formation. Segregation for morphological characters and high fertility in the F_2 and BC_1 indicate the possibility of gene exchange between these two species also.

Since unidirectional success is a common phenomenon in all crosses between any two species, cytoplasmic differentiation is assumed to have played the primary role in speciation.

Cytoplasm which prevents the development of a hybrid embryo can be considered to be an evolved cytoplasm, while cytoplasm which permits development of a hybrid embryo can be considered to be the original type. By this reasoning, mung bean would have more nearly the original cytoplasm, since this cytoplasm had by far the least inhibitory effect on hybrid embryos.

V. radiata var. soblobata (syn. Phaseolus soblobatus), which is a wild species and considered to be the progenitor of mung bean and black gram by Zukovskij (1962), is a likely common ancestor of these four species. The evolutionary relationships of these four species are postulated in Fig. 44.

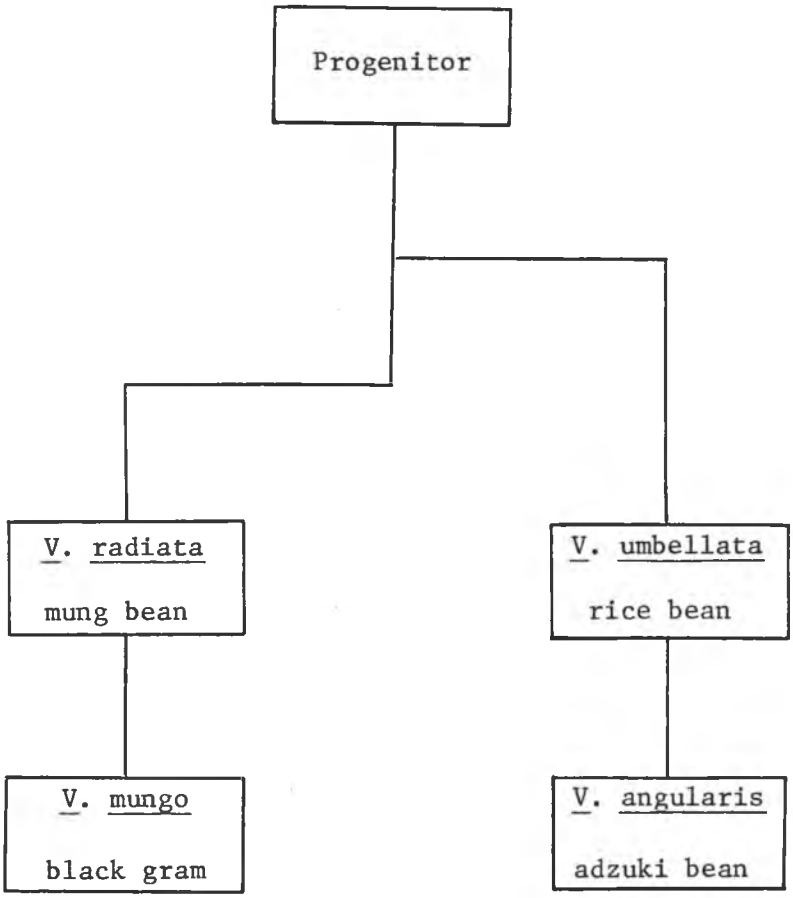

Fig. 44. Proposed evolutionary relationships of the four Vigna species.

Mung bean and rice bean might have been derived independently from this wild species, but the rice bean probably evolved first. Black gram and adzuki bean seem to be later derivatives from mung bean and rice bean, respectively. Thus, these four species can be divided into two subgroups, mung bean with black gram and rice bean with adzuki bean, in which the two species of each group retain enough chromosomal homology to allow germplasm exchange within the group.

This postulation is supported by the grouping of these four species into two taxa by Piper and Morse (1914) on the basis of morphological similarities.

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